UNIVERSITY OF LIVERPOOL

Studies on equine eosinophilic granuloma and mast cell tumour

Thesis submitted in accordance with requirements of the University of Liverpool for the degree of Doctor in Philosophy

by

Asma Mohamed Elbahi

September 2018

AUTHOR'S DECLARATION

Apart from the help and advice acknowledged, this thesis represents the unaided work of the author

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Asma Mohamed Elbahi

September 2018

This research was carried out in the Department of Veterinary Pathology and Public Health, Institute of Veterinary Science and Institute of Infection and Global Health, University of Liverpool

LIST OF ABBREVIATIONS

Ab	Antibodies
APC	Antigen presenting cells
CD3	Cluster of differentiation 3
CD79a	Cluster of differentiation/B-cell antigen receptor complex associated protein alpha chain
c-KIT	Proto-oncogene receptor tyrosine kinase
DAB	3,3′ diaminobenzidine
DAPB	Bacterial gene coding for dihydrodipicolinate reductase
DPX	Distyeren plasticiser xylene
EDTA	Ethylene diamine-tetra acetic acid
EEG	Equine eosinophilic granuloma
EG	Eosinophilic granuloma
EGC	Eosinophilic granuloma component
EOs	Eosinophils
Eotaxin/CCL11	C-C motif chemokine ligand 11
FFPE	Formalin-fixed paraffin-embedded
H	Hour
HC	Histochemistry
HE	Haematoxylin and eosin
HPF	High power field
Iba1	Ionised calcium-binding adapter molecule 1
IHC	Immunohistochemistry
IPPB	Housekeeping gene coding for peptidylprolyl isomerase B
ISH	In situ hybridisation
KIT	Protein product of KIT proto-oncogene receptor tyrosine kinase
MBP	Major basic protein
MCA	Mast cell aggregates
MCs	Mast cells
MCT	Mast cell tumour

Min	Minute
Mip1α/CCL3	Macrophage inflammatory proteins 1-alpha/chemokine (C-C motif) ligand 3
MPH	Macrophages
PCNA	Proliferating cell nuclear antigen
PPIB	Peptidylprolyl isomerase B
RANTES/CCL5	CC chemokine ligand 5
RT	Room temperature
SCF	Stem cell factor
TB	Toluidine blue
TGFβ	Transforming growth factor beta 1

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DEDICATION

I dedicate this thesis to my late father Mr Mohamed Al-Hashmi Elbahi (2 August 1931 – 23 August 2010, Tripoli, Libya), and to my mother Mrs Naima Mohamed Karrwa. To both of you, I am eternally grateful. You have made me the person I am today, and it is with your love, guidance and support that I have reached this milestone. You raised me to be strong and obedient. Everything you have done for me, has made me the person I am today. I am very proud that you are my parents. This is also dedicated to all my family (sisters, brothers, nieces and nephews).

And to All Libyan children, with love.

ACKNOWLEDGEMENTS

First and foremost, I would like to express my thanks and appreciation to Dr Lorenzo Ressel and Professor Anja Kipar for persevering with me during my studies in the Department of Veterinary Pathology of the University of Liverpool, in the United Kingdom.

As my primary supervisor, Dr Lorenzo Ressel took time to help me plan and organise my research and write my thesis. My inspiration for doing this research came from his plans, thoughts, advice, direction, feedback and support. I am very grateful for his kindness and encouragement at both personal and academic levels.

I would also like to express my gratitude and deep appreciation to my second supervisor, Professor Anja Kipar, who generously gave her time and expertise to improve my work. I am grateful for her plans, thoughts, advice, direction, feedback and support. It would have been impossible to complete this work without the supervision of Dr Ressel and Prof. Kipar. I am eternally grateful to both of them for the advice and help that I have received from them during the four years of my study.

I also wish to thank Professor Debbie Archer, who is currently based at Philip Leverhulme Equine Hospital and Department of Epidemiology and Population Health (Institute of Global Health, University of Liverpool) for her help. I would also like to thank my advisory panel: Dr Richard Blundell, Senior Lecturer in Veterinary Pathology and Dr Eleni Michalopoulou, Senior Lecturer in Veterinary Public Health, both at the University of Liverpool. I am grateful for their great advice and support relating to my work.

I would like to express my gratitude to all the technicians of the Histology Laboratory, Veterinary Laboratory Services, Department of Veterinary Pathology and Public Health, University of Liverpool, for their excellent technical support. Special thanks and gratitude to Valerie Tilston, who gave me assistance in the laboratory. Many thanks also to Dr Fernando Romero-Palomo, based at Institute of Veterinary Pathology, University of Zurich for his excellently performed RNA-ISH.

Special thanks to Dr Ranieri Verin for the encouragement, support and friendship he showed me during my studies at Leahurst. Thanks also to Mrs Patricia Jonker-Cholwe, Principal Library Assistance, School of Veterinary Science at Leahurst Campus for the friendship and hospitality she gave me throughout my entire study at Leahurst. Further, I would like to thank Dr Francisco Fernandez-Flores for his support and friendship, and Dr Emmanuel Ricci for his help and assistance. I would like to say a big thank you to Mr Tony Brandwood, Ms Marion Pope, Ms Elena Fitzpatrick and Ms Pippa Mahen for their support, encouragement and kindness

during my studies. I would like to say thank you to Dr Guido Rocchigiani for his help, support, and friendship.

I would also like to acknowledge many of my family, friends, colleagues, and fellow students who encouraged and assisted me in numerous ways. They advised and supported my research and writing efforts over the years.

Finally, I would like to thank the Ministry of Higher Education, Libya for granting me the scholarship and Tripoli University, the Faculty of Veterinary Medicine, for giving me the opportunity to do my PhD at one of the best well-known universities in the United Kingdom.

ABSTRACT

Eosinophilic granuloma (EG) and mast cell tumour (MCT) are skin diseases that affect horses as well as other animal species. In equine species they exhibit similar and potentially overlapping morphological features, and it is not clear if they are two different entities or represent a continuum of the same disease. Equine eosinophilic granuloma is the most common inflammatory nodular skin disease in horses and represents a chronic inflammation characterised by intense infiltrates of eosinophils associated with macrophages. The cause of this change is not fully clear. However, different factors are reported to potentially cause it and in turn lead to a hyper sensitivity reactions, dominated by eosinophils. In contrast, equine mast cell tumour is an uncommon neoplastic disease characterised by aggregates of mast cells, admixed with variable numbers of eosinophils and scattered areas presenting with lesions morphologically identical to those observed in equine eosinophilic granuloma. The aim of this study is to investigate the morphological and molecular features of equine eosinophilic granuloma and mast cell tumour in order to better understand their pathogenesis.

One hundred and ninety one lesions from 153 horses which had previously been diagnosed with equine eosinophilic granuloma or mast cell tumour were retrieved form the archive of the Department of Veterinary Pathology and Public Health, University of Liverpool. Clinical data were recorded and histomorphological features were investigated. The present study showed that Thoroughbreds with Thoroughbreds cross, and Arabian with Arabian cross are the most affected breeds with EG and MCT. In addition, however, a large number of different breeds were affected which had no sex predilection. With regard to MCT, the Arab breed was significantly predominant and males were overrepresented. The age range was wide, with older horses more affected by MCT than by EG, as expected. The most common location of the lesions was the trunk for eosinophilic granuloma and the head for MCT.

The morphological examination for 191 lesions was performed regardless (blind) of initial diagnosis. This analysis revealed variable histological features among cases. Criteria examined were: location of lesions within the skin, lesion size, area and number of eosinophilic granuloma component (EGC) and mast cell aggregates (MCA), presence of individual mast cells infiltrating the lesions, association between EGC and hair follicle structures, presence of embedded collagen in EGC, evidence of mineralisation, evidence of ulceration, density of infiltrating viable eosinophils within the MCA, mitotic activity of the mast cell population, and presence and number of lymphocyte aggregates. Using these criteria, four lesions exhibiting unique morphological features were identified. These cases were characterised by marked eosinophil infiltration and embedded mast cell population with very large pleomorphic mast cells. These four cases were excluded from the study and are described separately in Appendix II section-B.

From all 191 examined cases, 95 lesions were selected for further analysis. Five morphological categories were created based on the presence and relationship between the EGC and MCA components as following: category I (EGC only), category II (EGC > MCA), category III (EGC= MCA), category IV (EGC < MCA), and category V (MCA only). Based on these categories, three major groups were created: Group1 (Category I), Group2 (Categories II, III, and IV), and Group3 (Category V). The newly classified eosinophilic granuloma (EG) corresponded to roup 1 (Category I), while mast cell tumour (MCT) was identified through gGroups 2+3 (Categories II, III, IV, and V). EG lesions exhibited more variable histological features among cases within group 1, while MCT lesions were more similar among other groups. There were differences in lesion size and number between the categories with EG being smaller than MCT with size among different categories of MCT being highly variable. Deep dermis was the most common location for MCT, while EG was the lesion found most superficially. However, both EG and MCT stretched to different layers and when a "depth score" was evaluated, there was no difference between categories. Interestingly, a strong association between Category I lesions (EG) and hair follicle structures involvement was identified (52% of cases). Individual mast cells scattered throughout the lesions were evident in 13 lesions (42%) of Category I (EGC only) while they were present in all lesions of Category II (EG > MCT), and absent in the other categories. The analysis of Von Kossa stained sections indicated that EG exhibited significantly more mineralisation than MCT ($P<0.001$). Ulceration was present in both EG and MCT with no difference and was probably a secondary change not linked with the pathogenesis of either lesions. Collagen fibres or their fragments were visualised in almost all EG lesions (93%) within EGC, using the Masson Trichrome Stain. EG showed significantly more fragments of collagen within the EGC cores than MCT. The presence of lymphocytes aggregates was mainly a feature of EG (61% cases) rather than MCT, where only rare lymphocytes aggregates were detected in a few cases. The density of viable eosinophils infiltrating in MCA had an increasing trend among categories. The number of mitotic figures was different among Categories (II to V) with significantly higher numbers in Category V ($P<0.01$). Morphological evaluation identified macrophages actively phagocytosing eosinophils; this was only detected in MCT lesions, while no evidence of such phenomenon was noted in EG. Lendrum's stain, used to identify eosinophils within the sections, successfully highlighted the eosinophils granules, but also demonstrated that eosinophils in EGC cores exhibited different staining colours (purple, intense red, pale red) or were colourless. The presence of this colour pattern demonstrated that the EGC core did not have a regular centrifugal development as expected, but an "irregular onion ring" like arrangement, with different orders of colours change, overlapping each other. Purple Lendrum's stain overlapped perfectly with von Kossa stained areas suggesting that this chromatic affinity indicates mineralisation.

In the present study infiltration of immune cells was investigated using immunohistochemistry. CD3- positive cells, indicative for T lymphocytes were

infiltrating in large numbers in EG and MCT, but higher CD3 density was detected in EG lesions compared to MCT. CD79a-postive lymphocytes, indicative for B lymphocytes, which though rare in both MCT and EG lesions were, when present, forming lymphocyte aggregates. These cells were significantly more present in EG lesions compared to MCT ($P<0.05$). Histiocytes (Iba1- positive) were more numerous in EG compared to MCT; in both categories they were highly represented. Their observation was associated with EGC but also infiltrating in between mast cells in MCT, possibly representing tumour-associated macrophages (TAMs).

Analysis of cytokine expression was performed using *in-situ* hybridisation on formalin fixed paraffin embedded tissue (Eotaxin, MIP1-alpha, IL-4, IL-5, IL-13, TGF-beta, SCF) or immunohistochemistry (RANTES) in a sub group of lesions (n=10) belonging to EG (n=5) and MCT (n=5). In EG the predominant cytokine expressed by macrophages and lymphocytes including epithelioid macrophages was IL-13, which was significantly more expressed in EG compared to MCT. In MCT the most represented cytokine was IL-5 which was transcribed by mast cells and lymphocytes and was significantly higher in MCT than EG ($P<0.05$). Among other cytokines RANTES was expressed in lymphocytes and histiocytes in higher number in EG compared to MCT. Other cytokines investigated such as IL-4, MIP-1a, TGF beta, and SCF showed weaker differences amongst groups, and their role in the two lesions was less clear.

Summarising, according to the results of this study:

EG were predominantly lesions characterised by EGC with mineralised cores and the presence of collagen fragments. EGC were associated in some cases with lymphocytes aggregates composed of B cells, and often extended to the most superficial layers of the skin with hair follicle involvement. T cells were predominant, and IL-13 transcribed by lymphocytes and macrophages is likely to be predominantly involved in eosinophils recruitment in these lesions.

MCT were lesions predominantly located in deep dermis, exhibiting a variable number and variable sizes of EGC or, in some cases, none. MCT exhibited large numbers of infiltrating macrophages consistent with TAMs. Within the MCA, scattered macrophages phagocytosing eosinophils were detected, a unique feature of MCT. EGC associated with MCT were less often mineralised than those of EG, and only very rarely associated with B cells. IL-5 was the most abundant cytokine transcribed in these lesions by mast cells and lymphocytes, and it is believed that it is associated with the recruitment and increase in survival of eosinophils.

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CHAPTER 1: INTRODUCTION

1. Introduction

Eosinophilic granuloma (EG) is a chronic inflammatory condition observed in horses, cats and, though less frequently, dogs. It is characterised by intensive infiltrates of eosinophils, macrophages as well as lymphocytes and plasma cells (Ackermann, 2017, Mauldin and Peters-Kennedy, 2016). Despite its frequency, the aetiology and pathogenesis of EG is still unknown and possibly multifactorial (Mauldin and Peters-Kennedy, 2016, Scott and Miller, 2011b). In contrast, mast cell tumours (MCT) are neoplastic processes that involve the presence of neoplastic mast cell accumulations in a tissue. MCT are commonly reported in domestic animals, particularly in dogs and cats, but also occur in horses. The aetiology is unknown (Clarke *et al.,* 2014, Kiupel, 2017, Mair and Krudewig, 2008, Mauldin and Peters-Kennedy, 2016, Ressel *et al.,* 2015, Scott and Miller, 2011c).

However, different factors have been suggested to be associated with EMCT (equine mast cell tumour), such as parasitic infection, chronic inflammatory and autoimmune responses (Cole *et al.,* 2007, Malikides *et al.,* 1996). Interestingly, MCT and EG in horses share similar histological features, which often lead to misdiagnosis and confusion (Kiupel, 2017, Scott and Miller, 2011b, 2011c).

At present, it is not clear whether a "continuum" exists between the two processes (EG and MCT) in horses, or whether they are two different entities with an unrelated pathogenesis.

The association between inflammation and cancer as well as tumorigenesis has been discussed (Korniluk *et al.,* 2017, Landskron *et al.,* 2014, Porta *et al.,* 2009, Prete *et al.,* 2011, Taniguchi and Karin, 2018). In addition, the associated mechanisms and malignant transformation have been explored in laboratory animals (Erdman and Poutahidis, 2010, Maeda and Omata, 2008, Mantovani *et al.,* 2008). However, it is likely that a relation between chronic inflammation with or without infection and cancer exist and is similar in both humans and laboratory animals (Morrison, 2012). In fact, the number of reports documenting similarities in genetic expression, inflammatory cell infiltrates, cytokine and chemokine expression, and other

biomarkers in humans and domestic animals is continuously increasing (Morrison, 2012, Fürdös *et al.*, 2015, Carvalho *et al.*, 2016). So far, however, there is insufficient data to document the proportion of malignant tumours in domestic animals that result from inflammation (Morrison, 2012). In veterinary medicine, available information on inflammation and infection associated with cancer formation is scarce and predominantly limited to short and case reports (Ewald and Swain Ewald, 2015, Morrison, 2012). During active inflammation, the cell microenvironment is highly unstable with combined abundance of reactive oxygen and nitrogen species, cytokines, chemokines, and growth factors (Brenner *et al.*, 2014, Yang and Zhang, 2017). Any disturbance in normal tissues will trigger immune cells which results in an inflammatory response and unleashes various mediators such as cytokines, chemokines, matrix-remodelling proteases, reactive oxygen, and nitrogen species (Gabriele and Bruno, 2009, Roca *et al.*, 2009).

Ultimately, the relationship and link between inflammation and cancer development is well accepted (Crusz and Balkwill, 2015, Elinav *et al.*, 2013, Fernandes *et al.*, 2015, Korniluk *et al.*, 2017, Landskron *et al.*, 2014, Multhoff *et al.*, 2011), though many questions remain unanswered (Morrison, 2012).

1.2 Chronic inflammation, granulomatous inflammation, granuloma and eosinophilic granuloma: Concepts and cellular components

1.2.1 Chronic inflammation

Chronic inflammation is an inflammation of prolonged duration (Ackermann, 2017, Kumar *et al.*, 2017). It occurs when an acute inflammatory response has failed to eradicate the causative agent or following a repeated acute inflammation process leading to severe tissue injury and necrosis, or due to exposure to several factors (biochemical or virulence) in response to the inciting stimulus or microorganism (Ackerman, 2017). Chronic inflammation is the simultaneous occurrence of active inflammation, tissue destruction and attempts at repair (Kumar *et al.*, 2017). Chronic inflammation is defined morphologically by the presence of macrophages, lymphocytes and plasma cells in tissues (Ward, 2010). When inflammation becomes chronic, the adaptive immune response is activated with involvement of the cellular and non-cellular mechanisms of acquired immunity (Kumar *et al.*, 2017, Ward, 2010). Immune mechanisms play further roles in the resolution of

inflammation and in the healing process, including the repair and regeneration of lost and damaged tissues (Cekici *et al.*, 2014, Gilroy and De Maeyer, 2015, Headland and Norling, 2015, Sugimoto *et al.*, 2016).

In chronic inflammation, the response is predominantly regulated by cytokines, chemokines, and inflammatory mediators released by inflammatory cells at the inflammation site (such as macrophages, lymphocytes and plasma cells), leading to severe tissue damage, proliferation of fibroblasts, and the deposition of collagen (Ackermann, 2017, Headland and Norling, 2015, Kumar *et al.*, 2017).

1.2.2 Granulomatous inflammation

Granulomatous inflammation is a distinctive type of chronic inflammation, and it is characterised by infiltration dominance of macrophages including epithelioid macrophages (i.e. activated macrophages) and multinucleated giant cells (Ackermann, 2017, Kumar *et al.*, 2017, Shah *et al.*, 2017). The immune cells are arranged either in a random fashion or as organised nodules, i.e. granulomas (Ackermann, 2017). Different causative agents are responsible for granulomatous inflammation and include exogenous agents (infectious e.g. bacteria, viruses, fungi, protozoa, helminths and environmental substances - such pollens, dust mites, foodstuff) or endogenous antigens (e.g. autoantigens, tumour antigens, and viral antigens) and/or idiopathic factors (Ackermann, 2017, Kumar *et al.*, 2017). Several factors are required to develop and regulate granulomatous inflammation. These factors can be categorised as inciting agents, the host immune response (T cells and macrophages), interactions between and reaction of the infiltrating cells via cytokines, chemokines, and inflammatory mediators at the site of chronic inflammation (Ackermann, 2017, Kumar *et al.*, 2017).

1.2.3 Granuloma

A granuloma is a focus of chronic inflammation consisting of aggregations of macrophages that (including epithelioid macrophages and multinucleated gain cells) are surrounded by layers of lymphocytes and plasma cells (Kumar *et al.*, 2017).

A current definition of the term “granuloma” is provided by Pagán and Ramakrishnan (2018, p 639)

"Granulomas are organised aggregates of macrophages, often with characteristic morphological changes, and other immune cells. These evolutionarily ancient structures form in response to persistent particulate stimuli—infectious or non-infectious—that individual macrophages cannot eradicate".

1.2.4 Eosinophilic granuloma (EG)

Eosinophilic granuloma (EG) is a specific type of chronic inflammation characterised by intense infiltrates of eosinophils with macrophages, and variable amounts of lymphocytes and plasma cells (Ackermann, 2017). EG exhibits flame figures (dense eosinophilic area around collagens due to accumulation of Major Basic Protein (MBP) from degranulated and degenerated eosinophils) as noted by Ackermann, (2017), and Fondati *et al.,* (2001). EG sometimes do not form distinct nodules and the inflammatory cells (eosinophils and macrophages) are distributed in variable numbers randomly within the lesions (Ackermann, 2017). A definite cause has so far not been determined in any of the affected species (Ackermann, 2017, Mauldin and Peters-Kennedy, 2016).

1.3 Eosinophilic dermatitis in animals

Eosinophilic dermatitis is a frequent skin condition in several animal species and, in particular, in cats and horses (Bloom, 2006, Gross *et al.*, 2005, Mauldin and Peters-Kennedy, 2016). Eosinophilic inflammation is a common reaction in both cats and horses (Bloom, 2006, Gross *et al.*, 2005, Mauldin and Peters-Kennedy, 2016). It is also thought to be associated with those chemotactic stimuli that recruit neutrophils in other species, but eosinophils and neutrophils in cats and horses (Mauldin and Peters-Kennedy, 2016). Among eosinophilic skin inflammatory conditions in horses, EG represents a particular morphological entity (Scott and Miller, 2011b).

Several factors can induce eosinophilic dermatitis. These include environmental allergens, food, parasites, insects, drugs, endogenous (free keratin in dermis) and exogenous factors (for example, embedded insect parts), foreign material, and viral infections (feline herpesvirus 1 in cats) (Buckley and Nuttall, 2012, Mauldin and Peters-Kennedy, 2016).

Eosinophilic granuloma in cats, eosinophilic stomatitis in dogs and eosinophilic granuloma dermatitis in horses are possibly caused by antigen in T helper cells, however so far no specific antigen has been detected (Ackermann, 2017, Wobeser, 2015) .

1.3.1 Eosinophilic granuloma complex in cats

Feline eosinophilic granuloma complex encompasses a number of distinct clinical entities with overlapping histopathologic features (Buckley and Nuttall, 2012, Mauldin and Peters-Kennedy, 2016). These primary entities (namely feline eosinophilic granuloma, eosinophilic plaque and indolent ulcer) can occur separately or in combination, without breed or sex predilection (Bloom, 2006, Buckley and Nuttall, 2012). In addition, some studies such as that by Bonello *et al.*, 2012, have reported that eosinophilic granuloma complex is more common in young cats (2-4 years) and amongst females; but this possible predisposition is not well documented (Buckley and Nuttall, 2012).

It has been suggested that the above terms are used for clinical dermatology, whereas "Eosinophilic dermatosis" should be used in diagnostic dermatopathology (Bloom, 2006, Fondati *et al.*, 2001). The causative agent of feline eosinophilic granuloma complex is unknown, however, many causes have been suggested which assume that eosinophilic granuloma complex is the consequence of a hypersensitivity reaction to, amongst other things, insect bites wuch as fleas and mosquitos, atopy, and food allergies) (Bonello *et al.*, 2012, Mauldin and Peters-Kennedy, 2016). Other proposed causes are eosinophil dysfunction, embedded insects parts or foreign bodies, and the release of major basic protein (MBP) (Buckley and Nuttall, 2012). Studies in cats indicate that flame figures result from the deposition of eosinophilic MBP on collagen bundles and widespread degranulation of eosinophils and eosinophilic debris surrounding the collagens bundles (Fondati *et al.*, 2001, Bardagí *et al.*, 2003). In addition, the flies' domestic allergen I (FELD I) can be an auto-allergen responsible for chronic inflammation in felines with eosinophilic granuloma complex as noted by Hnilica, (2017), and Wisselink *et al.*, (2002).

Typically, indolent ulcer occurs as solitary, unilateral, alopecic, ulcerated, firm lesions on the upper lip adjacent to the philtrum, with possible associated regional

lymphadenopathy. These lesions are non-pruritic, non-painful, and rarely associated with peripheral eosinophilia (Bloom, 2006, Bonello *et al.*, 2012, Gross *et al.*, 2005, Mauldin and Peters-Kennedy, 2016). Eosinophilic plaques are typically raised, round, oozing, and erythematous and occur at the abdomen, thighs, inguinal and axillary areas with associated regional lymphadenopathy. They are severely pruritic with associated peripheral eosinophilia (Bloom, 2006, Gross *et al.*, 2005, Mauldin and Peters-Kennedy, 2016). In contrast, EG lesions of young cats show variable pruritus and possible eosinophilia and are either cutaneous or oral, papular, nodular or linear lesions. Cutaneous lesions are linear and appear at the caudal or medial thighs, as well as on footpads, the pinnae, and the lower lip. Oral lesions occur as nodules on the tongue, frenulum, or soft plate, and are more severe and aggressive than their cutaneous counterpart (Bloom, 2006, Bonello *et al.*, 2012, Forsythe, 2011, Gross *et al.*, 2005, Hnilica, 2017, Miller *et al.*, 2013).

Histologically, feline eosinophilic granuloma complex comprises dermal infiltrates dominated by eosinophils and is organised in various patterns (superficial, deep perivascular, interstitial, or diffuse), with or without flame figures, and may exhibit small foci of collagen fibres expanded by oedema and surrounded by degranulated eosinophils. In addition, a granulomatous reaction may be present around the flame figures (Fondati *et al.*, 2001, Bardagí *et al.*, 2003). The most common histological features of indolent ulcer are eosinophilic infiltration with fewer neutrophils and variable fibrosis, with rare eosinophilic degranulation around collagen fibres (flame figures). In eosinophilic plaque, severe epithelial hyperplasia with acanthosis, spongiosis and eosinophilic exocytosis, as well as epidermal and follicular mucinosis are common findings; eosinophils may infiltrate the panniculus, and ulceration is often observed (Bloom, 2006, Gross *et al.*, 2005, Mauldin and Peters-Kennedy, 2016).

Feline EG is seen as diffuse dermal granulomatous inflammation, with numerous eosinophils, fewer mast cells and macrophages, and sometimes lymphocytes. In the inflamed areas, there are large irregular foci of eosinophilic degranulation and degeneration around collagen bundles (flame figures). These large foci can be surrounded by macrophages and multinucleated giant cells. Focal infiltrative to necrotising mural eosinophilic folliculitis or furunculosis, and focal eosinophilic

panniculitis may be seen in cutaneous lesions. In addition, the overlying epithelium is often hyperplastic and ulcerated, and mucinosis of the epidermis and hair follicle's outer root sheath may be present. Older lesions often have palisading macrophages around flame figures, with fewer eosinophils (Buckley and Nuttall, 2012, Mauldin and Peters-Kennedy, 2016). The differential diagnosis for eosinophilic granuloma complex in cats includes neoplasia (squamous cell carcinoma, lymphoma, mast cell tumour), dermatophytosis, feline cowpox infection, cutaneous viral disease, mycobacterial infection, deep fungal infection, bacterial folliculitis and furunculosis or abscess, foreign body reaction, sterile granulomatous disease, and trauma-induced changes (Bloom, 2006, Buckley and Nuttall, 2012, Gross *et al.,* 2005).

1.3.2 Canine eosinophilic granuloma

Canine eosinophilic granuloma is a rare condition and shares many histological features with feline eosinophilic granuloma (Bloom, 2006, Bonello *et al.*, 2012, Gross *et al.,* 2005, Hnilica, 2017, Kim *et al.*, 2011, Mauldin and Peters-Kennedy, 2016). An age or sex predisposition is not reported (Vercelli *et al.,* 2005). Nevertheless, EG has been reported as more common in young male Siberian Huskies and Cavalier King Charles (Bredal *et al.,* 1996). The cause of canine eosinophilic granuloma is unknown. However, genetic factors and a hypersensitivity reaction are suggested, along with multiple allergen factors are proposed (Bredal *et al.*, 1996, Kim *et al.,* 2011, Mauldin and Peters-Kennedy, 2016).

Clinically, EG lesions in dogs are mainly nodules or plaques in the oral passage, associated with blood eosinophilia (Bonello *et al.*, 2012, Kim *et al.,* 2011, Mauldin and Peters-Kennedy, 2016). Cutaneous lesions are less common and present as non-pruritic lesions at different body sites such as the muzzle, neck, flank and prepuce (Vercelli *et al.,* 2005). The typical histological finding is diffuse dermal eosinophilic inflammation, commonly found as foci of bright eosinophilic collagen surrounded by degranulating eosinophils and occasionally surrounded by epithelioid macrophages and possibly ulcerated or acanthotic epithelium/epidermis (Bloom, 2006, Gross *et al.*, 2005, Mauldin and Peters-Kennedy, 2016). Differential diagnosis for canine eosinophilic granuloma are an infectious and foreign body

granuloma, neoplasms and eosinophilic furunculosis (Bloom, 2006, Gross *et al.*, 2005).

1.4 Equine eosinophilic nodular diseases

Eosinophilic inflammation is a common reaction pattern in horses. There are three nodular conditions of unknown aetiology and pathogenesis, including equine eosinophilic granuloma (EEG), axillary nodular necrosis and unilateral papular necrosis (Mauldin and Peters-Kennedy, 2016, Scott and Miller, 2011b). Hypersensitivity reaction, insect bites, silicone-coated needles are suspected causes of such inflammatory reactions (Scott and Miller, 2011b, Slovis *et al.*, 1999). In addition, there are other nodular diseases in horses, with a defined aetiological cause, which share histological features with EEG, i.e. are characterised by massive eosinophilic infiltration and areas of necrosis; for example, cutaneous habronemiasis. Furthermore, cutaneous mast cell tumours can share both clinical and histological features with equine eosinophilic granuloma (EEG) and are also of unknown aetiology (Mauldin and Peters-Kennedy, 2016, Scott and Miller, 2011c).

1.4.1 Equine eosinophilic granuloma (EEG)

Equine eosinophilic granuloma (EEG) is the most frequent inflammatory nodular skin disease and cutaneous eosinophilic nodular condition in horses (Pilsworth and Knottenbelt, 2005, Scott and Miller, 2011b, White, 2015, Wobeser, 2015). It is reported worldwide and without breed, sex, or age association (Mauldin and Peters-Kennedy, 2016, Pilsworth and Knottenbelt, 2005, Scott and Miller, 2011b, Wobeser, 2015). Previously, these conditions were termed "collagenolytic granuloma" which turned out to be inaccurate because several studies demonstrated that the areas of brightly eosinophilic collagen surrounded by eosinophils do not represent areas of collagen degradation but are "flame figures" as described above (Ackermann, 2017, Mauldin and Peters-Kennedy, 2016, Scott and Miller, 2011a).

1.4.1.1 Pathogenesis and causes of equine eosinophilic granuloma (EEG)

Despite the frequency of EEG, its aetiology and pathogenic mechanisms are still unknown. EEG is likely multifactorial, and several factors have been thought to cause and trigger such an inflammatory reaction, including traumatic injury in the

saddle region and atopy (Mauldin and Peters-Kennedy, 2016, Scott and Miller, 2011b, Wobeser, 2015).

1.4.1.2 Clinical findings

EEG generally presents as single or multiple nodular lesions from 0.5-10 cm in diameter and are most frequently found on the withers and back, in the girth area, the mane, the rump, and the face (Mauldin and Peters-Kennedy, 2016, Scott and Miller, 2011b, Wobeser, 2015). The lesions are often round, firm and well circumscribed, without ulceration, alopecia, pain or pruritus. Some lesions, however, can be cystic or plaque-like, with a central caseous core (Mauldin and Peters-Kennedy, 2016, Scott and Miller, 2011b, Wobeser, 2015). The key histopathologic feature of EEG is the collagen degeneration/collagenolysis or necrobiotic collagen. However, it is currently known as flame figures, due to the presence of eosinophilic degranulation products released from degranulated eosinophils coating the collagen but there is no collagen degeneration (Fernandez *et al.*, 2000, Scott and Miller, 2011b).

1.4.1.3 Diagnosis and histopathological findings

History and clinical signs as well as skin biopsy are used to diagnose EEG (Scott and Miller, 2011). In addition, the cytological examination shows infiltration of different immune cells (such as eosinophils, histiocytes, and lymphocytes) and the absence of microorganism (Mathison, 1995, Scott and Miller, 2011b). The typical histological finding of EEG is a nodular to diffuse eosinophilic granulomatous inflammation of the dermis, occasionally also the panniculus.

Multifocal areas of “flame figures” are a key feature, as well as occasional small foci of folliculitis or furunculosis and the presence of scattered lymphoid nodules, which could, in some cases, predominate in the lesions. In advanced lesions, there are areas of dystrophic mineralisation, which can lead to misdiagnosis as calcinosis circumscripta (Mauldin and Peters-Kennedy, 2016, Scott and Miller, 2011b, Wobeser, 2015).

The differentiation of EEG from unilateral papular necrosis and axillary nodular dermatosis is relatively straightforward; however, these two lesions have many

features in common with EEG which may lead to diagnostic challenges (Mathison, 1995, Stannard, 2000).

EEG needs to be differentiated form other eosinophilic nodular skin diseases including parasitic diseases such as habronemiasis (summer sore), fungal diseases such as pythiosis and zygomycosis, neoplastic diseases such as mast cell tumours, and multisystemic eosinophilic epitheliotropic diseases (MEED) (Scott and Miller, 2011b).The latter is a rare eosinophilic condition that results in eosinophilic infiltration with occasional eosinophilic granuloma formation in many organs, including the skin and intestine (Scott and Miller, 2011b).The cause of MEED is considered either ato be severe hypersensitivity reaction or a clonal proliferation of T cells, resulting in the recruitment of eosinophils due to the secretion of interleukin 5 (Laisse *et al.*, 2017, Perle *et al.*, 1998).

1.4.2 Unilateral papular dermatosis

Unilateral papular dermatosis is an uncommon nodular skin disease in horses and reported worldwide especially in spring and summer. Some studies suggest that the quarter horse breed is over-represented (Mathison, 1995, Mauldin and Peters-Kennedy, 2016, Scott and Miller, 2011b, Stannard, 2000). The cause and pathogenesis is still unknown. However, due to the eosinophilic nature of the lesion and the seasonality, it has been proposed that it could represent an insect bite hypersensitivity reaction (Scott and Miller, 2011b, Stannard, 2000). Since the lesions occur on one side of the body, a hypersensitivity reaction to an ectoparasite inhabiting the bedding would also be a possibility (Scott and Miller, 2011b). Clinically, the condition is characterised by multiple unilateral cutaneous papules of 2-10 mm in size, preferentially on the trunk but sometimes on the neck, shoulders or abdomen, with no signs of pruritus or pain. However, some lesions become crusted and alopecic. Histologically, the lesions are characterised by eosinophilic folliculitis and furunculosis, and eosinophilic granulomas with flame figures may be present in the surrounding dermis (Mathison, 1995, Mauldin and Peters-Kennedy, 2016, Scott and Miller, 2011b, Stannard, 2000). Some lesions can exhibit wedge-shaped areas of necrosis or superficial to mid dermal eosinophilic granulomas and necrosis but without clear involvement of hair follicles (Scott and Miller, 2011b).

1.4.3 Axillary nodular necrosis (girth galls)

Axillary nodular necrosis is a very rare dermatosis of horses that can occur throughout the year with no age, breed, or sex predilection (Mauldin and Peters-Kennedy, 2016, Scott and Miller, 2011b). Cause and pathogenesis is still unclear. This dermatosis is also described as a focal nodular eosinophilic granuloma and arteritis (Mauldin and Peters-Kennedy, 2016, Scott and Miller, 2011b). The condition, also known by owners as "girth galls", is linked to overworking or ill-fitting tack. However, it has been reported in both working and nonworking horses (Scott and Miller, 2011b, Stannard, 2000). Clinically, it is characterised by cutaneous nodules that typically occur in the girth area, or behind the elbow. However, it may also appear caudal to the shoulder and on the proximal, medial aspect of the forearm (Mauldin and Peters-Kennedy, 2016, Scott and Miller, 2011b, Stannard, 2000). The lesions are firm, round, well circumscribed, and 1-10 cm in diameter, can be single or multiple and are sometimes arranged in a linear pattern; with neither pruritus nor pain. The overlying skin and hair coat are usually unaltered. Histologically, the lesions are characterised by coagulative necrosis surrounded by eosinophilic granulomatous dermatitis, usually with panniculitis, flame figures, dystrophic mineralisation of collagen, lymphoid nodules, eosinophilic or necrotising vasculitis with endothelial cell hypotrophy, vessel occlusion, and intimal mucinosis (Mauldin and Peters-Kennedy, 2016, Scott and Miller, 2011b).

1.5 Mast cell tumours in animals

Mast cell tumours (MCT) occur in many animal species such as dogs, cats, horses and cows at diverse locations and with variable biological behaviour; similarities out-weigh variations (Goldschmidt and Hendrick, 2002).

MCT in animals frequently affect the dermis and subcutis as focal or multicentric nodular masses (Goldschmidt and Hendrick, 2002, Kiupel, 2017, Misdorp, 2004), but can in principle occur in many different tissues and organs, such as the gastrointestinal tract, respiratory tract, salivary glands, eyes, testes and spleen (Head *et al.,* 2002, Kiupel, 2017, Mair and Krudewig, 2008).

Among domestic animals, MCT have been most frequently reported and most intensely studied in dogs, as well as in cats while they have only been infrequently or sporadicly reported in horses (Kiupel, 2017, Mair and Krudewig, 2008, Misdorp, 2004).

In dogs, MCT are the most common malignant skin cancer and exhibit significant variability in their biological behaviour. They develop most frequently in the skin, but can also grow in the intestines, liver, spleen, and elsewhere (Kiupel, 2017). Cutaneous MCT comprise 7-21% of skin tumours in dogs, with no sex or age predilection, however many breeds are predisposed, the boxer being the best known example (Blackwood *et al.,* 2012, Kiupel, 2017, Strefezzi *et al.,* 2009). So far, knowledge and information on the differences between cutaneous and subcutaneous MCT are sparse. Nonetheless, previous studies have shown that subcutaneous MCT are generally less aggressive than their cutaneous counterparts (Blackwood *et al.*, 2012, Kiupel, 2017, Thompson *et al.,* 2011). In addition, extracutaneous MCT or mast cell leukaemia are rarely seen (Strefezzi *et al.,* 2009).

In cats, mast cell tumours are the second most common cutaneous tumours, accounting for approximately 20% of skin tumours. Siamese cats appear to be predisposed to MCT, but other breeds can also be affected, and there is no sex predisposition (Blackwood, 2015). Feline MCT (FMCT) is classified histologically into Mastocytic and Atypical.

Mastocytic tumours can be further classified into 2 subtypes: well differentiated MCT and pleomorphic MCT (Blackwood, 2015, Blackwood *et al.,* 2012, Goldschmidt and Hendrick, 2002, Sabattini and Bettini, 2010). Well differentiated MCT are completely circumscribed encapsulated masses that can extend into the subcutis. They are mostly composed of sheets of cells resembling normal mast cells, with mild pleomorphism, a low mitotic rate and low numbers of eosinophils and lymphocytes (Goldschmidt and Hendrick, 2002, Gross *et al.,* 2005, Sabattini and Bettini, 2010).

Pleomorphic MCT on the other hand exhibit a tendency to infiltrate deep into the dermis and subcutis (Gross *et al.,* 2005, Sabattini and Bettini, 2010). They are comprised of large cells with eccentric nuclei and prominent 1`nucleoli, and contain

variable numbers of giant cells with a multilobulated nucleus. Regarding the mitotic rate, there are conflicting results; an older study reported a variable and often low mitotic rate (Johnson *et al.*, 2002), whereas a more recent one describes high mitotic activity (Sabattini and Bettini, 2010). Pleomorphic MCT are usually infiltrated by large numbers of eosinophils (Hendrick *et al.*, 1998, Goldschmidt and Hendrick, 2002, Gross *et al.*, 2005). Recently, a new subcategory of well differentiated MCT with prominent multinucleated cells has been described (Melville *et al.*, 2015).

Poorly differentiated tumours carry a worse prognosis; but most feline cutaneous MCT (50-90%) are well differentiated and show benign behaviour (Blackwood, 2015). The atypical form of MCT occurs primarily in Siamese cats under 4 years of age. Siamese cats are also predisposed to the development of the mastocytic form. This type of FMCT previously termed "histiocytic" is characterised by large, polygonal to spindle shaped neoplastic cells with abundant amphophilic cytoplasm. The nuclei are large and vesicular and may be slightly indented; mitotic figures are infrequent. Admixed eosinophils and lymphoid aggregates tend to be more numerous than in mastocytic MCT (Blackwood, 2015, Henry and Herrera, 2013, Sabattini and Bettini, 2010).

In cattle, mast cell tumours are infrequent and can affect many organs too. However, the skin is the most frequently affected site and usually develops malignant and highly metastatic MCT resulting in multicentric cutaneous disease (Kiupel, 2017, Pérez *et al.*, 1999, Smith and Phillips, 2001). Young animals can exhibit solitary or multiple lesions at various body sites (Hill *et al.*, 1991, Kiupel, 2017, Shaw *et al.*, 1991, Smith and Phillips, 2001, Welle *et al.*, 1995). Furthermore, congenital systemic mast cell tumours have also been reported in a stillborn calf (Smith and Phillips, 2001).

In horses, mast cell tumour is an uncommon neoplastic disease and understanding of it is limited (Goldschmidt and Hendrick, 2002, Kiupel, 2017, Mair and Krudewig, 2008, Scott and Miller, 2011c). Equine mast cell tumour (EMCT) is characterised by aggregate areas of neoplastic mast cells, admixed with variable numbers of eosinophils, and areas of eosinophilic granuloma and dystrophic mineralisation (Kiupel, 2017, Mair and Krudewig, 2008, Scott and Miller, 2011c).

The latter are similar to those seen in EEG, and frequently lead to misdiagnosis of EMCT (Kiupel, 2017, Mair and Krudewig, 2008, Scott and Miller, 2011c).

1.5.1 Histopathology and grading classification systems of MCT in animals

In all other species, apart from dogs, and to a lesser extent cats, there is limited information on their morphological classification (Mair and Krudewig, 2008). However, in dogs MCT has been widely studied and described. Several classification and grading systems based on the degree of cellular differentiation, the nuclear morphology and the overall cellularity have been proposed (Bostock, 1973, Hottendorf and Nielsen, 1967, Patnaik *et al.*, 1984).

The Patnaik grading system (Patnaik *et al.*, 1984) is the best known grading scheme and is used to predict the clinical behaviour of MCT in dogs; it divides canine MCT into three grades. More recently, the Patnaik system was criticised for its inaccuracy regarding certain categories (i.e. MCT grade 2) (Kiupel *et al.,* 2004), and due to its ambiguity, leading to discordance (50%) among pathologists in grading MCT (Northrup *et al.,* 2005, Pinczowski *et al.,* 2008). A recent two-tier system for grading MCT in dogs was developed based on the number of mitoses, the presence of multinucleated cells or bizarre nuclei, and karyomegaly (increased nuclear size) (Kiupel *et al.*, 2011).

High-grade MCT tumours have been shown to be significantly associated with a shorter time to metastasis, tumour associated mortality, and a shorter overall survival time (less than 4 months). Low-grade MCT tumours, on the other hand, have a median survival time of more than two years (Kiupel *et al.*, 2011).

Furthermore, several recent studies have demonstrated that subcutaneous MCT in dogs should be distinguished from their cutaneous counterparts, and that the existing grading systems are not appropriate for their prognosis (Blackwood *et al.*, 2012, Garrett, 2014, Thompson *et al.,* 2011). Notably, more than 90% of subcutaneous MCT have a benign course and can be controlled surgically (Kiupel, 2017, Newman *et al.*, 2007, Thompson *et al.,* 2011). In addition, 10% of dogs with subcutaneous MCT die from MCT-associated diseases whilst 11% develop a second subcutaneous MCT distant from the first one. Moreover, 5-8% recur and metastasise, respectively (Garrett, 2014, Kiupel, 2017, Thompson *et al.*, 2011).

1.6 Mast cell tumours in horses

In horses, MCT represent 3-7% of the referred cutaneous neoplasms cases in equine hospitals (Scott and Miller, 2011c, Valentine, 2006). So far, they have not been fully investigated and most of our knowledge is based on a limited number of studies as well as other descriptive reports in individual animals.

The classification of MCT in horses is also referred to as "mastocytosis" or "mastocytoma" (Altera and Clark, 1970, Cheville *et al.,* 1972, Mair and Krudewig, 2008, Mauldin and Peters-Kennedy, 2016, Moulton, 1990). It is still debated (Altera and Clark, 1970, Clarke *et al.,* 2014, Goldschmidt and Hendrick, 2002, Johnson, 1998, Kiupel, 2017, Mair and Krudewig, 2008, Mauldin and Peters-Kennedy, 2016, Millward *et al.,* 2010, Moulton, 1990, Prasse *et al.,* 1975, Ressel *et al.,* 2015, Scott and Miller, 2011c). Moreover, an aetiology has not been identified, but parasitic infections, chronic inflammation and autoimmune reactions have been reported to be associated with equine MCT (Cole *et al.,* 2007, Malikides *et al.,* 1996).

Equine MCT can be misdiagnosed as an inflammatory skin disease (such as eosinophilic granuloma), due to shared common features including: the variable sizes of mast cell aggregates, the well -differentiated appearance of neoplastic mast cells, the high number of infiltrating eosinophils, the well-circumscribed areas of collagen degeneration (flame figures) and focal to multifocal areas of necrosis, and the level of mineralisation (Goldschmidt and Hendrick, 2002, Kiupel, 2017, Mair and Krudewig, 2008, Mauldin and Peters-Kennedy, 2016, Scott and Miller, 2011c, Tan *et al.,* 2007).

In addition, older (present for a long time) cutaneous MCT usually exhibit a variable degree of fibrosis and palisading granuloma formation and can also display extensive dystrophic mineralisation similar to that found in eosinophilic granulomas (Goldschmidt and Hendrick, 2002, Scott and Miller, 2011c). It follows, that MCT may share characteristics with calcinosis criumscripta (Goldschmidt and Hendrick, 2002, Mair and Krudewig, 2008, Mauldin and Peters-Kennedy, 2016, Scott and Miller, 2011c).

1.6.1 Clinical sings of equine mast cell tumour

Equine MCT are generally benign lesions. They occur in three different clinical forms; solitary, congenital, and a malignant form affecting many organs (Mair and Krudewig, 2008). Solitary tumours are the most common form, affecting mainly the superficial skin (Mauldin and Peters-Kennedy, 2016, Ressel *et al.*, 2015, Scott and Miller, 2011c).

Solitary cutaneous MCT usually show one of two notable patterns; the slowly progressive or static growth, where chronic lesion is rarely shows sudden rapid growth (Kiupel, 2017, Mair and Krudewig, 2008, McEntee, 1991, Scott and Miller, 2011c). The cutaneous MCT lesions are most frequently single, but multiple lesions widely separated or close to each other may occasionally present in the skin of horses (Altera and Clark, 1970, Mair and Krudewig, 2008, McEntee, 1991).

Solitary MCT affects the dermis or subcutis of the head (i.e. lip, nostril, jaw, and periorbital area), trunk, and limbs. It usually presents as a partially encapsulated and firm or fluctuant lesion. Other features such as alopecia, hyperpigmentation or ulceration can also be found (Mair and Krudewig, 2008, Scott and Miller, 2011c). MCT usually present as non-painful and nonpruritic lesions, but may exhibit sporadic discharge of caseous material (Mair and Krudewig, 2008). In contrast, MCT that affects other areas such as the limbs are predominantly firm and immovable, and tend to be found in proximity to joints. In these latter locations, portions of the tumour are usually calcified and may be visible radiographically (Samii *et al.*, 1997).

The congenital form of cutaneous mastocytosis is rare in horses and mainly affects newborn foals and spontaneously regress. In this way it resembles human cutaneous mastocytosis in young children (Mair and Krudewig, 2008, Prasse *et al.*, 1975). This form is characterised by multifocal cutaneous nodules of variable size, occurring on the trunk, lateral hind limbs and within bone marrow (Prasse *et al.*, 1975).

Malignant MCT are the rarest in horses (Malikides *et al.*, 1996, Martin and Leipold, 1972, Reppas and Canfield, 1996, Riley *et al.*, 1991, Ritmeester *et al.*, 1997). The reported cases that exist describe a primary intraosseous tumour, which only

affected the phalanx, synovial infiltration and can spread to local lymph nodes. It may lead to lameness but without documented involvement of other organs. In addition, the malignant form is associated with circulating eosinophilia, hyperfibrinogenaemia; furthermore, pruritus associated with the lesion or disseminated pruritus have also been reported (Reppas and Canfield, 1996, Riley *et al.*, 1991).

Widespread metastasis appears to be extremely rare (Mair and Krudewig, 2008). However, *Tan et al*., (2007) described a multicentric form of mastocytoma, which affected the thoracic and abdominal cavities as well. Furthermore, multicentric MCT have been reported in a Rocky Mountain horse which exhibited primary clinical signs such as draining tracts and distal limb subcutaneous oedema involving multiple limbs (Millward *et al.*, 2010).

Equine MCT have been also reported in the eye, affecting the conjunctiva, sclera, nictitating membrane and globe (Doran and Collins, 1986, Flores *et al.*, 2017, Halse *et al.*, 2014, Hum and Bowers, 1989, Lavach, 1992, Malikides *et al.*, 1996, Martin and Leipold, 1972, McEntee, 1991, Nyrop, 1992, Ward *et al.*, 1993). They have also been described in nasal cavities and the nasopharynx (Malikides *et al.*, 1996, McEntee, 1991, Richardson *et al.*, 1994), the trachea (Wenger and Caron, 1988), and the tongue (Seeliger *et al.*, 2007).

To date there is no clear evidence of any risk factors associated with MCT in horses, however it has been suggested that the male sex is predisposing (Altera and Clark, 1970, Doran and Collins, 1986, Mair and Krudewig, 2008, McEntee, 1991, Samii *et al.*, 1997, Stannard, 1976). In addition, Arab horses appear to be over-represented (Clarke *et al.*, 2014, Cole *et al.*, 2007, Mair and Krudewig, 2008, Samii *et al.*, 1997).

1.6.2 The prognosis of equine mast cell tumours

To date there is limited information pertaining to the typing and grading of equine cutaneous MCT. Two recent investigations have described recurrent and multicentric tumours and investigated the morphological characteristics of cutaneous MCT in horses; they obtained contrary results (Clarke *et al.*, 2014, Ressel *et al.*, 2015).

Clarke *et al.* investigated the histologic features and KIT expression patterns of 72 equine cutaneous MCT in order to determine any associations with clinical outcomes. The study concluded that most tumours were well differentiated with low mitotic rates (i.e. 96%), and normal KIT staining patterns. It concluded that KIT staining pattern and histologic features are not associated with clinical outcome. In contrast, Ressel *et al.,* investigated 45 equine MCT and assessed the proliferation (PCNA expression) and KIT expression together with morphological features. They identified two types of MCT, well differentiated and poorly differentiated. Following the definition of the KIT expression patterns in dogs (Webster *et al.,* 2004), most well differentiated MCT were found to exhibit KIT pattern I, whereas one third (comprised of poorly differentiated mast cells) showed KIT pattern II or III. The authors showed that the aberrant KIT expression accompanies a high proliferation rate and that these two features characterise a subset of poorly differentiated equine MCT. This confirmed their true neoplastic nature (Ressel *et al.,* 2015). This latter study supports the need for further investigations to understand the biological process of cutaneous MCT in horses.

1.7 Diagnostic and prognostic methods of MCT in animals

Diagnostic and prognostic assessment approaches to cutaneous MCT have been a focus of research over a longer period in particular in dogs and on a combination of different methods leading to a variety of guidelines (Table 1). Generally, these methods are based on history, clinical sings, cytological and/or histological examination (Blackwood, 2015, Scott and Miller, 2011c, Strefezzi *et al.,* 2009). Furthermore, the behaviour and progression of cutaneous MCT is highly variable, leading to treatment failure in a number of cases. However, several methods have been developed and used particularly in the treatment of pets (Blackwood *et al.,* 2012, Strefezzi *et al.,* 2009).

Table 1: Overview of published approaches for the diagnosis of MCT in animals

Method	Basis of diagnosis	Specific and main feature of confirmation	Animal species	Reference
Cytology	Fine needle aspiration Wright's stain	Large numbers of mast cells associated with eosinophils (in dogs and horses, no eosinophils in FNA of cats)	Dog, cat, horse	(Blackwood, 2015, Friedrichs and Young, 2013, Kiupel, 2017, Pavel *et al.,* 2016)
Histological examination	HE and Toluidine Blue stain	Aggregates of neoplastic mast cells/ metachromatic reaction for mast cells granules	Dog, cat, horse	(Blackwood, 2015, Kiupel, 2017, Scott and Miller, 2011)
Histological grading system	Histological criteria	Well differentiated and poorly differentiated	Dog	(Kiupel, 2017, Patnaik *et al.*, 1984)
IHC	PCNA, KIT Ki-67	Positive reaction	Dog, horse	(Kiupel, 2017, Ressel *et al.*, 2015, Webster *et al.*, 2007)

1.7.1 Diagnostic approaches to mast cell tumours

1.7.1.1 Cytological examination

A rapid diagnosis of cutaneous MCT can be achieved through a cytological evaluation of fine needle aspirates (FNA) or the impression smears of excisional surgical biopsies (Blackwood *et al.,* 2012, Friedrichs and Young, 2013, London and Seguin, 2003). Typical MCT produce a smear with large numbers of mast cells associated with eosinophils. However, mast cell granules might not be easy to identify with the rapid modified Wright's staining techniques due to the aqueous nature of the granule contents that may not be metachromatic (Friedrichs and Young, 2013, Jackson *et al.,* 2013, London and Seguin, 2003, Raskin and Meyer, 2016).

These are effective methods for initial diagnosis in some animals and limit the list of differential diagnoses (Friedrichs and Young, 2013, Pavel *et al.,* 2016). However, it is recommended that there is a need to confirm the cytological assessment though histology, using HE and Toluidine Blue-stained sections (Kiupel, 2017, London and Seguin, 2003).

1.7.2 Prognostic assessment

1.7.2.1 Histological grading systems

For canine MCT, a combination of the histological grading systems with additional approaches are combined (Amorim *et al.,* 2010, Fonseca-Alves *et al.,* 2015, Murphy *et al.,* 2004, Patnaik *et al.,* 1984). This does not exist for MCT within other species.

1.7.2.2 Mitotic index

The mitotic index (MI) is based on the determination of the number of mitoses /10 high power fields (Blackwood *et al.,* 2012, Romansik *et al.,* 2007, Strefezzi *et al.,* 2009). For instance, a median survival estimation for dogs suffering from MCT and having a MI of less than 5 was 70 months, whereas for an MI of 5 or more it was 2 months (Blackwood *et al.,* 2012, Romansik *et al.,* 2007). In addition, dogs with MCT showing a MI≤10 had a median survival of approximately 11 weeks only (Blackwood *et al.,* 2012, Romansik *et al.,* 2007). The impact of MI on the survival index has recently been a topic of discussion (Kiupel, 2017).

1.7.2.3 Histochemical and immunohistochemical approaches

1.7.2.3.1 Cell proliferation markers

Evaluation of the proliferative activity using cell proliferation markers aims to complement or substitute the counting of mitoses (Stoimenov and Helleday, 2009). Proliferating cell nuclear antigen (PCNA) is a non-histone protein necessary for DNA synthesis and repair. It is maximally expressed during the S-phase of the cell cycle (Bologna-Molina *et al.,* 2013, Stoimenov and Helleday, 2009); this high expression is associated with recurrent and metastatic tumours (Mukaratirwa, 2005, Romansik *et al.,* 2007, Scase *et al.,* 2006).

AgNORS are argyrophilic nucleolar organising region-associated proteins that bind silver molecules and can be identified using histochemical staining methods (Scase *et al.,* 2006, Simoes *et al.,* 1994). By counting the number of AGNOR dots per nucleus hasbeen used to demonstrate the generation time (speed of cell cycle progression). AgNOR counts were correlated with survival times but did not allow

for the prediction of clinical behaviour independent of histological grade (Blackwood *et al.,* 2012, Strefezzi *et al.,* 2009).

Ki-67 (also known as MKI67) is a cellular protein marker of proliferation. During the interphase, Ki-67 antigen can be exclusively detected within the cell nucleus, whereas in mitosis most of the protein is relocated to the surface of the chromosomes. Conversely, during G0 Ki-67 is not detectable. As a consequence of this it is considered to be a reliable marker of proliferation. Moreover, it generally strongly correlates with the degree of tumour malignancy (Dziegiel *et al.,* 2005). In canine MCT, detection of KI-67 in association with cKIT is a recommended and routine prognostic method (Fonseca-Alves *et al.,* 2015, Poggiani *et al.,* 2012, Webster *et al.,* 2007).

1.7.2.4 Oncogenes

Genes involved in neoplastic transformation are derived from proto-oncogenes that regulate normal cellular growth. Several oncogenes have been described; growth factor receptors are often over expressed in cancer (Hayes, 2012).

The expression of the proto-oncogene cKIT has been extensively studied in MCT; it encodes a transmembrane receptor with tyrosine-kinase activity which binds with Stem Cell Factor (Blackwood *et al.,* 2012, Strefezzi *et al.,* 2009). This factor, also named the Mast cell growth factor or KIT-ligand, is a cytokine that activates the maturation and survival of mast cells as well as other cells such as haematopoietic stem cells and melanocytes (Blackwood *et al.,* 2012, Costa Casagrande *et al.,* 2015, Strefezzi *et al.,* 2009).

Mutation and genetic deletion within the juxtamembrane domain are responsible for the constitutive activation of the receptor and such activation leads to excessive cell proliferation and tumour formation (Blackwood *et al.,* 2012, Strefezzi *et al.,* 2009).

Mutations were detected and described as juxtamembrane domain duplications in MCT in dogs (Strefezzi *et al.,* 2009). In 2000 Reguera *et al.* reported higher KIT levels as the tumour differentiation decreased and two immunohistochemical staining patterns: membranous or cytoplasmic, usually around the nucleus. Another

study detected mutations in MCT grades II & III and no mutations in grade I (Zemke *et al.*, 2002). This study also proposed that KIT mutations and expressions can be associated with mast cell tumorigenesis and progression. A further study by Marconato *et al.*, (2014) suggested that c-KIT mutation is associated with increased metastasis and local recurrence as well as a higher histological grade.

Kiupel *et al.* (2004) suggested a new classification for canine cutaneous MCT based on the immunohistochemical KIT staining patterns. Three distinct patterns were described: (I) membrane associated staining, with minimal cytoplasmic staining; (II) intense focal or stippled cytoplasmic staining; and (III) diffuse cytoplasmic staining, obscuring other cytoplasmic features. The results of this research showed KIT-II and KIT-III patterns to be significantly associated with local recurrence and shorter survival times in dogs (Kiupel *et al.*, 2004).

There is limited information on prognostic approaches for local recurrence and/or metastasis of canine subcutaneous MCT. However, Thompson *et al.* (2011) have demonstrated that a panel of markers (KIT immunohistochemical labelling pattern, c-Kit mutational status (presence of internal tandem duplications in exon 11), and proliferation markers including MI, Ki-67 and AgNOR) can be used to assess the behaviour of subcutaneous MCT in dogs.

1.8 Prognosis of equine cutaneous mast cell tumours

In horses, the diagnosis of MCT is mainly based on histopathology and cytology (Cole *et al.*, 2007, Scott and Miller, 2011c). The variable histopathological appearance of both benign and malignant MCT in large animals suggests that the canine classification system cannot be applied (Halse *et al.*, 2014, Hill *et al.*, 1991, Riley *et al.*, 1991, Ritmeester *et al.*, 1997, Tan *et al.*, 2007). Indeed, one recent study concluded that there are no reliable diagnostic tools to characterise the different biological behaviour of equine MCT (Clarke *et al.*, 2014). In contrast, Ressel *et al.* (2015), found that certain differentiation features, proliferative activity and c-Kit expression identify true, i.e. neoplastic MCT and that c-KIT expression might be a prognostic tool for cutaneous equine MCT.

1.9 Role, involvement and interaction of immune cells in eosinophilic granuloma

Eosinophils, macrophages and, to a lesser extent, lymphocytes, are major players in inflammation and inflammatory reactions in EG lesions (Ackermann, 2017). In chronic inflammation, macrophages and T lymphocytes are the main immune cells, which release various cytokines, chemokines and proteins causing damage to living cells. They are also responsible for the initiation of cell-mediated immunity. Other cells are also involved in such immune responses, i.e. mast cells, eosinophils, and neutrophils (Murphy and Weaver, 2017, Tizard, 2013). Given this, the current chapter focuses on the interaction of eosinophils, macrophages, lymphocytes and mast cells, and is predominantly based on human literature.

1.9.1 Eosinophils

Eosinophils are multifunctional immune cells that originate from pluripotent progenitors in bone marrow and differentiate under the influence of cytokines such as interleukin (IL) 5, IL-3 and granulocyte-macrophages colony stimulating factor (GM-CSF) (Rosenberg *et al.*, 2012). Eosinophils play a key role in the pathogenesis of numerous inflammation processes, microbial infections, and allergic reactions as well as immunoregulatory responses (Eng and DeFelice, 2016, Kita, 2011, Kita, 2013, Long *et al.*, 2016, Muniz *et al.*, 2012, Rosenberg *et al.*, 2012) .Their granules are also responsible for inflammation and tissue damage and are well known as the primary source of inflammatory mediators in type 1-hypersenstivtiy as well as the release of toxic substances by degranulation. Such a mechanism is responsible for combating parasitic infections (Kita, 2011, Kita, 2013, Long *et al.*, 2016, Parker and Papenfuss, 2017, Stone *et al.*, 2010). Furthermore, eosinophils can phagocytose small materials (yeast cell walls, foreign erythrocytes, and antigen–antibody precipitates), and has been demonstrated in horses (Archer and Hirsch, 1963). In addition, human eosinophils have been reported to phagocyte bacteria (Cline *et al.*, 1968, Shamri *et al.*, 2011), as well as phagocytose and kill large parasites (Mauldin and Peters-Kennedy, 2016, Shamri *et al.*, 2011). Furthermore, eosinophils produce transforming growth factor (TGF)-β which is frequently correlated with chronic inflammation (Halwani *et al.*, 2011, Mauldin and Peters- Kennedy, 2016, Ohno *et al.*, 1992, Yang *et al.*, 2012). At the site of chronic inflammation, both pro-

inflammatory cytokines and chemokines work in coordination and attract mature eosinophils to the site (Barnes, 2008, Ghosh *et al.*, 2013). Numerous mediators can induce chemotactic and/or chemokinetic processes and are known to induce eosinophil migration (Ghosh *et al.*, 2013). The initiation as well as the maintenance of these migrant eosinophils depends on the nature of the signals. Receptors and receptor profiles on eosinophils also dictate which chemoattractants regulate mobility and induced movement. The promotion of receptors and the localisation of downstream signalling mediators as well as the binding of IL-5, IL-3 and GM-CSF regulate and prepare eosinophil responses to chemoattractants and the initiation of mobility-movement by the chemotactic agent (Gregory *et al.*, 2003, Simson and Foster, 2000, Uhm *et al.*, 2012).

Furthermore, eosinophil recruitment and production is largely due to the stimulation of Th2 lymphocytes, as well as numerous cytokines (i.e. IL-3, IL-4, IL-5, IL-13), and GM-CSF (Davoine and Lacy, 2014, Kita, 2013, Kita, 2011, Long *et al.*, 2016). The primary function of IL-4 and IL-13 is to stimulate the production of immunoglobulin (Ig) E which promotes eosinophil recruitment by increasing the expression of eotaxin (CCL11 and CCL26) and endothelial cell vascular cell adhesion molecule 1 (VCAM1) (Stone *et al.*, 2010).

In contrast, IL-5 mediates the increase of eosinophil production, eosinophil mobility from bone marrow, and the activation and survival of eosinophils (Kouro and Takatsu, 2009, Kita, 2011, Long *et al.*, 2016). Moreover, IL-5 is a cytokine specific for eosinophil development and IL-5 receptor expression as well as for eosinophil differentiation (Takatsu, 2011). In addition, extravasation via the endothelium is mediated by a system of molecules that function in chemotaxis and adhesion. These include α4 (CD49d), β2 (CD18) integrins and CC chemokines (CCL11/eotaxin) (Günther *et al.*, 2011).

The CC chemokine family is essential for eosinophil-enhanced-trafficking and infiltration to inflamed areas. As eosinophils highly expressing CCR3, a receptor that binds eosinophil specific chemokines including eotaxin, eotaxin-2, monocyte chemoattractant protein (MCP3, MCP-4), and regulated on activation, normal T cell expressed and secreted (RANTES or CCL5) (Weller, 2013, Ying *et al.*, 1999).

The way of eosinophils trafficking to inflamed tissues is possibly monitored by T cells responding to antigen presenting cells (Eng and DeFelice, 2016).

During apoptosis, eosinophils express phosphatidylserine on their plasma membrane, which leads to the recognition and engulfing of eosinophils by macrophages (Farahi *et al.,* 2011, Haslett, 1999, Stern *et al.,* 1992). Failure of the macrophages to clear apoptotic eosinophils can lead to secondary necrosis and the release of eosinophil granule proteins as well as other pro-inflammatory mediators (Farahi *et al.,* 2011, Stern *et al.,* 1992).

1.9.2 Macrophages

Macrophage are major immune cells which play a central role in inflammation and the initiation of immune responses (Hao *et al.,* 2012, Oishi and Manabe, 2016, Tizard, 2013, Ackermann, 2017). They represent the first line of defence and are the cells that come into contact with foreign invaders (Parisi *et al.,* 2018). In addition, they are capable of chemotaxis (Fernando *et al.,* 2014, Hao *et al.,* 2012). This is attributed to their toll-like and scavenger receptors, which have broad ligand specificity for lectins, lipoproteins, proteins, oligonucleotides, polysaccharides, and other molecules (Duque and Descoteaux, 2014, Hao *et al.,* 2012).

Macrophages originate from stem cells of the granulocytic–monocytic lineage in bone marrow, and are exposed to certain cytokines (Ackermann, 2017, Tizard, 2013). Their differentiation is associated with the expression of specific cytokine receptors, which are also essential for their function (Sieweke and Allen, 2013). Macrophages also mediate the unleashing of effective immune responses, link both innate and adaptive immunity and influence the microenvironment of cells (Duque and Descoteaux, 2014, Huynh *et al.,* 2007). Such influential interaction has been reported in inflammatory and tumour lesions, which provides diverse signalling interactions that divert macrophages into “classically activated” (M1) or “alternatively activated” (M2) types (Duque and Descoteaux, 2014, Edholm *et al.,* 2017).

In addition, macrophages express major histocompatibility complex (MHC) class II molecules on their surface, and present antigens to lymphocytes (Mantegazza *et al.,* 2013, Suzuki and Luo, 2017). When macrophages engulf a microbe, its antigens

are processed and situated on the outer surface of the plasma membrane, where they will be recognised by T helper cells (Mantegazza *et al.,* 2013). Following this recognition, T lymphocytes release cytokines that activate B cells, and activated B lymphocytes, then secrete antibodies specific to the antigens presented by the macrophages (Murphy and Weaver, 2017). These antibodies attach to antigens on microbes, or to cells invaded by microbes; in turn, these antibody-bound complexes are phagocytosed more avidly by macrophages (Duque and Descoteaux, 2014, Lacy, 2015, Tizard, 2013).

Macrophages can be activated in different ways by a variety of stimuli. Their activity is increased by cytokines that are released by T helper cells as well as interferon (IFN)-γ (Martinez and Gordon, 2014, Tizard, 2013). Classic (M1) macrophages are involved in defensive processes against pathogens whereas the alternative (M2) cells contribute to tissue remodelling (Italiani and Boraschi, 2014, Parisi *et al.,* 2018, Sica *et al.,* 2008). The polarisation is similar to that of lymphocytes as M1 cells are generated from monocytes in response to IFN-γ while M2 cells develop in response to cytokines such as IL4 and IL-13 (Fernando *et al.,* 2014, Foucher *et al.,* 2013, Mantovani *et al.,* 2013, Parisi *et al.,* 2018, Smith *et al.,* 2016). Macrophages are released in response to different kinds of chemokines such as CCL2, which in humans is responsible for their polarisation to M2 macrophages (Roca *et al.,* 2009). However, such described polarisation does not restrict these cells' range of existent phenotypes or the different related signalling factors required from the environment that lead to such range and variety (Galdiero *et al.,* 2014, Hao *et al.,* 2012, Parisi *et al.,* 2018). There are many aspects of polarised M1 and M2 macrophages, which are reflected in multiple functional phenotypes, (i.e. parasite infections, allergic reactions, and tumours), and are expressed by the macrophages both in vivo and vitro (Parisi *et al.,* 2018, Sica and Mantovani, 2012). The plastic nature of macrophages provides the shifts in the polarisation states of M1 and M2 during pathological courses (Biswas and Mantovani, 2010, Parisi *et al.,* 2018).

Lymphocytes are the main regulator of the function and activity of tumour-associated macrophages (TAMs); however, the pathways are variable in different organs (Sica and Mantovani, 2012). For example, a switch of macrophage function

was found to be mediated and influenced by IL-4 of Th2 cells in the skin (Andreu *et al.*, 2010, Schioppa *et al.*, 2011), and by antibody-producing B cells in breast cancer in humans (DeNardo *et al.*, 2009, Pedroza-Gonzalez *et al.*, 2011). Cancer progression can be promoted by B1 cells and macrophage polarisation, and tumour promotion can be regulated by fibroblasts (Biswas and Mantovani, 2010). In addition, other cell products in tumour tissues can also reshape macrophages in an M2-like cancer mode due to extracellular matrix components, IL-10, CSF-1, and various chemokines (e.g. CCL2, CCL18, CCL17, and CXCL4) (Sica and Mantovani, 2012).

1.9.3 T lymphocytes

T cells regulate adaptive immunity and are responsible for cell-mediated immune responses. T cells are generated in bone marrow, mature in the thymus and are activated in peripheral lymphoid organs (Day and Schultz, 2014, Murphy and Weaver, 2017, Tizard, 2013). They can be distinguished from other lymphocytes by the T cell receptor (TCR). This specific receptor is a heterodimer and most T cells express the α and β chains which recognise the peptide antigen only when it is bound in the context with a class I or class II MHC molecule. Further, the TCR connects with a complex of membrane proteins known as CD3 that are responsible for spreading an intracellular signal to TCR ligation. Moreover, each TCR connects with either a CD4 or a CD8 co-receptor, depending on the type of T cell. These two molecules bind to MHC (class I for CD8 and class II for CD4), which leads to further stabilisation of the interaction between T cells and antigen presenting cells (APC) (Ackermann, 2017, Covacu *et al.*, 2016, Day and Schultz, 2014, Miles *et al.*, 2011, Murphy and Weaver, 2017, Tizard, 2013).

In addition, α and β chains have three variable sites in the receptors known as complementarity determining regions (CDRs). These regions are immunoglobulin (Ig) hypervariable domains that determine specific antibody binding (Day and Schultz, 2014, Murphy and Weaver, 2017, Polonelli *et al.*, 2008, Tizard, 2013). However, other T cells (approx. 5%) express the γδ combination (Tizard, 2013).

T cell activation is mediated by TCR to produce a T cell that is capable of mediating immune protection via activation of naïve T cells. This involves coordinated interactions between a number of molecules on the T cell and an APC. An APC

(i.e. a macrophage or dendritic cell) detects, engulfs and digests pathogens into hundreds or thousands of antigen fragments. These are then transported to their surface as antigenic peptides and bound to a MHC class I or class II molecule. This, in turn, leads to the activation of naïve T cells and the initiation of their differentiation process (Day and Schultz, 2014, Murphy and Weaver, 2017, Tizard, 2013).

T cells differentiate into effector and/or memory cells after encountering their cognate antigen. The effector cells are further characterised as helper T cells that regulate immune responses, cytolytic or cytotoxic T cells that destroy cells expressing endogenous antigens, and regulator T cells that control the process (Chen and Kolls, 2013, Day and Schultz, 2014, Kara *et al.*, 2014, Mahnke *et al.*, 2013, Murphy and Weaver, 2017, Tizard, 2013).

The helper T cells (characterised by the expression of CD4 marker on their surface), secrete cytokines in response to antigenic stimulation. The latter stimulate the differentiation of T cells into various lineages and phenotypes, as well as other cells, including B cells and macrophages (Luckheeram *et al.*, 2012, Pennock *et al.*, 2013). There are three major subpopulation of T helper cells (Th1, Th2 and Th17). Th1 cells are stimulated by IL12 and secret (INF-y) and promote cell-mediated response. Th2 (IL4, IL13 and IL10) secrete and promote antibody responses. In contrast, Th17 are stimulated by IL6, TGF-β and IL23 to develop and secrete IL17 and promote neutrophil mediated inflammation (Day and Schultz, 2014, Murphy and Weaver, 2017, Tizard, 2013).

Cytolytic or cytotoxic T cells are characterised by the expression of CD8 marker on their surface and kill cells that are infected and/or produce foreign antigens, such as viruses and other intracellular microbes (Chen and Kolls, 2013, Murphy and Weaver, 2017, Sharma *et al.*, 2013, Zhu *et al.*, 2010).

The critical immune role of T cells is also in their functional helper role to B cells and to antibody responses, feedback to dendritic cells and elaboration of cytokines (Luckheeram *et al.*, 2012, Pennock *et al.*, 2013, Murphy and Weaver, 2017). They also enhance and maintain responses of CD8+ (cytotoxic) T cells and play a cytotoxic and defence role by mediating macrophage activation and inducing

associated genes (Flaherty, 2012, Luckheeram *et al.,* 2012, Pennock *et al.,* 2013, Murphy and Weaver, 2017).

Migrating lymphocytes are important regulators for efficient immune responses. T cell trafficking is the first and initial response. In this process, naive T cells migrate through the specialised endothelium of secondary lymphoid organs and the primed T cells exert their function by infiltrating through capillary venules into the tissues and to their designated antigenic site (Carman and Martinelli, 2015, Oelkrug and Ramage, 2014). In addition, activation and differentiation of T cells into effector or memory lymphocytes induces the expression of specific receptors (Zhu *et al.*, 2010). Such migration processes from peripheral blood into tissue include various processes and mechanisms that include: tethering, rolling and adhesion followed by diapedesis through the endothelial barrier which lines the inner wall of blood vessels (Nourshargh and Alon, 2014, Schnoor *et al.*, 2015, Slaney *et al.,* 2014). Chemokines are typically involved in the recruitment of lymphocytes and their secretion by the tissue, and they have been proven to recruit certain T cells (Oelkrug and Ramage, 2014). The chemokine environment plays a pivotal role in controlling lymphocyte trafficking (Stein and Nombela-Arrieta, 2005).

CCR5 ligands, CCL5 and macrophage inflammatory proteins (MIP-1α) are reported to be synthesised by activated dendritic cells and induce CXCR3 expressions on activated lymphocytes (Oelkrug and Ramage, 2014). CCR5 and CXCR3 chemokine receptors may play a pivotal role in the regulation of leukocyte migration to inflammatory sites (de Oliveira *et al.,* 2014).

1.9.4 B lymphocytes

B cells play a key role in inflammation and are essential in humoral and adaptive immunity (Chaplin, 2010, Murphy and Weaver, 2017, Tizard, 2013). They are generated and matured in bone marrow and differentiated in the spleen and lymph nodes into naïve, follicular, or marginal zone B cells (Murphy and Weaver, 2017, Pieper *et al.*, 2013, Tizard, 2013). The marginal zone B cells are partially activated along macrophages and dendritic cells, and exposed to antigens present in the blood. They provide a rapid humoral response to any challenges (Cerutti *et al.*, 2013, Zouali and Richard, 2011, Muppidi *et al.*, 2011). Marginal B cells contribute early in adaptive responses and, following activation, form short-lived plasma cells

that secrete antibodies (Cerutti *et al.,* 2013, Hoffman *et al.,* 2016, Murphy and Weaver, 2017, Tizard, 2013, Zouali and Richard, 2011).

B cells become active when they encounter an antigen (internalise and process), and thence present the latter to a perfectly matched T-helper cell, which then assist in the activation of the B cell (Hoffman *et al.,* 2016, Murphy and Weaver, 2017, Tizard, 2013). They are activated by many antigens, especially monomeric proteins. The process requires both binding of the antigen by the immunoglobulin on the B cell surface, the B-cell receptor, and interaction of the B cell with antigen-specific helper T cells (Avalos and Ploegh, 2014, Murphy and Weaver, 2017, Tizard, 2013). During the differentiation of activated B cells, the antibody isotype can change in response to cytokines released by helper T cells, and the antigen-binding properties of the antibody can be changed by the somatic hypermutation of V-region genes (Schroeder Jr *et al.,* 2013). Somatic hypermutation and selection for high-affinity binding occurs in the germinal centres of lymphoid follicles (Martin *et al.,* 2015). Helper T cells control these processes by selectively activating cells that have retained their specificity for the antigen and by inducing proliferation and differentiation into plasma cells and memory B cells. Some non-protein antigens stimulate B cells in the absence of recognition by peptide-specific helper T cells (Bortnick and Allman, 2013). These thymus-independent antigens induce only limited isotype switching and do not induce memory B cells (Hoffman *et al.,* 2016, Lange *et al.,* 2012). Nevertheless, responses to such antigens have a major role in host defence mechanisms against pathogens with antigens unable to elicit peptide-specific T-cell responses. Peptide fragments of the antigen, which is internalised and displayed by the B cells as peptide-MHC class II complexes, are typically recognised by helper T-cells (Murphy and Weaver, 2017, Tizard, 2013). Furthermore, B cells are induced and stimulated by the helper T-cells through the binding of CD40L on the T cell into CD40 on the B cell, the interaction of some TNF-TNF-receptor family ligand pairs, and by cytokines (Hoffman *et al.,* 2016, Murphy and Weaver, 2017, Upadhyay *et al.,* 2014). The initial interaction occurs in the T cell area of the secondary lymphoid tissue, where both antigen-specific and helper T cells and antigen-specific B cells are trapped as a consequence of binding antigen; further interactions between T cells and B cells occur after migration into the B cell zone or follicle, and the formation of a germinal centre (Murphy and

Weaver, 2017, Zhang *et al.*, 2016). Helper T cells induce a phase of vigorous B cell proliferation and direct the differentiation of the clonally expanded progeny of the naive B cells into either antibody-secreting plasma cells or memory B cells. Helper T cells control these processes by selectively activating cells that have retained their specificity for the antigen and by inducing proliferation and differentiation into plasma cells and memory B cells (Elgueta *et al.*, 2009, Hoffman *et al.*, 2016, Murphy and Weaver, 2017).

1.9.5 Mast cells

Mast cells are a particular component of the immune system and play a major role in inflammatory and immediate allergic reactions, and acquired and innate immunity (Beghdadi *et al.*, 2011, Dahlin and Hallgren, 2015, Gri *et al.*, 2012, Metz *et al.*, 2007, Tizard, 2013).

Mast cells are situated in the skin and mucosa under normal circumstances and are localised in connective tissue. They develop and differentiate in bone marrow and mature upon stem cell factor (SCF) binding to the receptor (c-kit) and various interleukins (IL-3, IL4, IL-9, and IL-10). The latter also enhance their differentiation and proliferation (Ackermann, 2017, Amin, 2012, da Silva *et al.*, 2014, Dahlin and Hallgren, 2015, Krystel-Whittemore *et al.*, 2015, Tizard, 2013).

Mast cells are the only immune cells that express the SCF (c-Kit) receptor, the binding of which enhances the release of histamine and tryptase proteins that are particularly involved in allergic responses and the promotion of adhesion, migration, proliferation, and survival of mast cells (Amin, 2012, Theoharides *et al.*, 2012, Tizard, 2013).

During inflammation, mast cells secrete inflammatory mediators (i.e. histamine, proteases, chemotactic factors, cytokines and metabolites of arachidonic acid) that act on the vasculature, smooth muscle cells, connective tissue, mucous glands and various leukocytes (da Silva *et al.*, 2014). Mast cells also play a biovital role in controlling and regulating the immune system function and associated mechanisms such as inflammation, cell activation, cell migration, cell proliferation, apoptosis, and haematopoiesis (Abraham and St John, 2010, Ackermann, 2017, Amin, 2012). Mast cells are particularly involved in chronic inflammatory conditions of the skin;

their activation and release of effective mediators is linked to various factors (Beghdadi *et al.,* 2011, Theoharides *et al.,* 2012). Mast cells can also express cell surface ligands and receptors, which can be either proinflammatory or immunosuppressive (Galli *et al.,* 2011). They can also recruit other immune cells such as T cells, neutrophils and eosinophils to the site of inflammation and stimulate the maturation of Langerhans cells and dendritic cells as well as their migration to lymph nodes (Gri *et al.,* 2012, Palm *et al.,* 2016). In addition, mast cells can migrate to draining lymph nodes and activate immune cells there (Amin, 2012, Harvima and Nilsson, 2011). They show remarkable plasticity in expressing different cytokines and TNF family ligands in skin inflammatory diseases (Abraham and St John, 2010, Galli *et al.,* 2011, Harvima and Nilsson, 2011, Oskeritzian, 2015). Furthermore, mast cell–macrophage interactions are involved in tumour biology through releasing mediators that affect the production of collagenolytic enzymes by metastatic tumour cells, tumour-derived fibroblasts and stromal fibroblasts (Dabbous *et al.,* 1995, Trivedi *et al.,* 2013).

1.10 Cytokines and chemokines in inflammation

Cytokines play a vital role in regulating immune responses (primarily inflammation) in both health and disease (Coondoo, 2011). Cytokines are small proteins that act as signalling and regulatory molecules at picomolar or nanomolar concentrations to regulate inflammation and biological activities such as growth, survival, and cellular differentiation (Duque and Descoteaux, 2014, Coondoo, 2011). Cytokines are diverse pro- or anti-inflammatory factors that are grouped into families based on receptors and structural homology (Coondoo, 2011). Chemokines are secreted proteins which are responsible for the induction of cell migration as well as being involved in chemoattraction and the trafficking of immune cells towards a site of injury and/or throughout the body (Duque and Descoteaux, 2014). Chemokines express particular biological activities by maintaining homeostasis and the induction of inflammation (Moser and Loetscher, 2001); based on their functions, two groups are recognised; homeostatic and inflammatory chemokines. Homeostatic chemokines are involved in immune cell migration through haematopoiesis and are constitutively expressed, whereas inflammatory chemokines are released in infections and/or as inflammatory stimulus and

contribute to the immune response process by targeting cells of the innate and adaptive immune system (Duque and Descoteaux, 2014, Chen *et al.,* 2018, Ferretti *et al.,* 2014, Sokol and Luster, 2015).

The binding of a cytokine or chemokine to its cognate receptor results in the activation and initiation of signalling events which regulate numerous cellular activities such as cell adhesion, phagocytosis, cytokine secretion, cell activation and proliferation, cell survival and cell death, apoptosis and angiogenesis (Coondoo, 2011, Geeta *et al.,* 2013). Cytokines have functional redundancy where different cytokines share similar functions (Duque and Descoteaux, 2014). They are also pleiotropic as they can act on many different cells, and such cells may express more than one receptor for each cytokine (Dinarello, 2007, Khan, 2008). Cytokines are generally short-lived and act locally in an autocrine and paracrine fashion. Few cytokines are present in the blood that contributes to a systemic response – examples include erythropoietin (EPO), TGF-β, and monocyte colony stimulating factor (M-CSF) (Dinarello, 2007, Tizard, 2013). Cytokines are mainly produced by macrophages and lymphocytes as well as polymorph nuclear leukocytes (PMN), endothelial and epithelial cells, adipocytes, platelets, and vascular smooth muscle cells (VSMCs) (Duque and Descoteaux, 2014, Sprague and Khalil, 2009).

Chemokines induce the migration of cells, such as lymphocytes, dendritic cells and macrophages (Ferretti *et al.,* 2014, Sokol and Luster, 2015). Chemokines can also be involved in both immune and physiological trafficking of leukocytes (Ferretti *et al.,* 2014, Stein and Nombela-Arrieta, 2005). However, certain chemokines are crucial inflammatory components of the microenvironment of tumours and play a pivotal role in their progression by enhancing the migration of tumour cells to distant organs (Raman *et al.,* 2007, Song *et al.,* 2017). Chemokines may also play a role during development by promoting angiogenesis, or by guiding cells to tissues that provide critical signals for a given cell's differentiation (Coondoo, 2011). In inflammation and inflammatory responses chemokines are released by a variety of cells involved in both innate and adaptive immunity together with the typical release of chemokines induced by proinflammatory cytokines such as TNF, IL-6, and IL-1β (Arango Duque and Descoteaux, 2014, Comerford and McColl, 2011).

1.11 Mediators associated with inflammation and inflammatory responses, with a focus on the interaction between mast cells, eosinophils and macrophages

During inflammation the immune cells secret inflammatory mediators (cytokines and chemokines), which play the most important role in controlling immune system functions (Ackermann, 2017, Day and Schultz, 2014, Murphy and Weaver, 2017, Tizard, 2013).

Different inflammatory and regulatory molecules mediate inflammation and guide inflammatory responses. Of these, CCL5 also known as RANTES (Regulated upon Activation, Normal T cell Expressed, and Secreted) is a chemokine regulatory activator expressed normally by T cells as well as macrophages, eosinophils and mast cells (Aldinucci and Colombatti, 2014, Lacy, 2017). CCL5 plays a key role in recruiting many leukocytes into inflammatory sites. It is also chemotactic for T cells, macrophages, eosinophils and basophils (Bishara, 2012, Lv *et al.*, 2013). It also has an effect in cancer cell proliferation, metastasis, and the formation of immunosuppressive microenvironments (Aldinucci and Colombatti, 2014).

Macrophage inflammatory protein MIPα (CCL3) is another chemokine that can be secreted by mast cells, macrophages and eosinophils and attracts eosinophils, macrophages and some lymphocyte subpopulations to sites of inflammation (Baba and Mukaida, 2014, Murphy and Weaver, 2017). CCL3 is produced by mast cells, and can attract monocytes, macrophages, and neutrophils (Murphy *et al.*, 2012). Together with other chemokines (CCL7, CCL13, CCL3, Rantes and eotaxin) it is responsible for the migration of eosinophils (Shakoory *et al.*, 2004). Many T cell chemoattractants are produced by mast cells including CCL3, CCL4 and CCL5, which can activate the endothelium appropriately for recruiting T cells (Marshall, 2004). Recruitment of eosinophils from the blood stream during inflammation and allergic processes was thought to be regulated by eotaxin and other chemokines including; CCL13, Rantes, eotaxin2, CCL3, and CCL7 (Schröder and Mochizuki, 1999, Ying *et al.*, 1999).

In addition, eotaxin is synthesised by different cells (e.g. epithelial cells, macrophages, T cells, eosinophils and mast cells) and is the most potent

chemoattractant for eosinophils (Conroy and Williams, 2001, Jia *et al.,* 1999). It also attracts other cells such as mast cells and some T cell subsets (Uguccioni *et al.,* 1997). In addition, IL-4, IL-13 and the proinflammatory cytokine tumour necrosis factor (TNF)-α stimulate the generation of eotaxin (Atasoy *et al.,* 2003, Conroy and Williams, 2001, Sato *et al.,* 2004), whereas IFN-γ inhibits the production of eotaxin (Sato *et al.,* 2004).

IL-4 is mainly secreted by some T cells, mast cells and eosinophils and plays a pivotal role in the transmobility of eosinophils across the endothelium through the activation of adhesion pathways. IL-4 can also promote the production of other cytokines such as IL-5 and eotaxin (Sato *et al.,* 2004) as well as inducing eosinophil chemotaxis and increasing the capacity of eosinophils to produce granule proteins (Atasoy *et al.,* 2003). IL-4 and similarly IL-3 are synthesised by mast cells and can stimulate and amplify Th2 responses (Metcalfe, 2008, Wynn, 2003).

IL-5 is mainly produced by T cells and mast cells (Murphy *et al.,* 2012) and is necessary for the growth and selective differentiation of eosinophils (Ghosh *et al.,* 2013, Murphy *et al.,* 2012). IL-5 enhances the growth and differentiation of activated B cells, however, its overexpression increases the quantity of eosinophils and elevates antibody levels (Takatsu and Nakajima, 2008, Takatsu, 2011). IL-5 also induces eosinophil differentiation in bone marrow as well as enhancing the stimulation of eosinophil precursors to produce granule proteins (Rosenberg *et al.,* 2012). It is also responsible for the mobilisation and release of differentiated eosinophils from bone marrow into the bloodstream and, through so doing, increases the number of circulating eosinophils as well as their responsiveness to chemoattractants and activators.Whilst also inhibiying the apoptosis of eosinophils, thereby increasinging their survival in tissues for weeks (Rankin *et al.,* 2000, Rosenberg *et al.,* 2012, Shen and Malter, 2015).

IL-5 is also important for the activation of eosinophils and may play a role in tissue infiltration by eosinophils; it shows functional synergy with eotaxin (Rosenberg *et al.,* 2012, Shakoory *et al.,* 2004). Mast cells are a good source of IL-5 and eosinophil-attracting chemokines (e.g. CCL5 (Rantes) and CCL11 (eotaxin) (Marshall, 2004). Mast cells also drive the survival of eosinophils through the release of GM-CSF and IL-5 (Brown *et al.,* 2008).

A few cytokines (IL3, IL5 and GM-CSF) are important for signalling molecules produced by T cells (Asquith *et al.,* 2008, Guthridge *et al.,* 1998). Of these, IL-5 is most specifically responsible for the selective differentiation of eosinophils (Rothenberg and Hogan, 2006, Shen and Malter, 2015, Wen and Rothenberg, 2016).

T cells also secrete IL-13, which is considered functionally redundant with IL-4. However, some studies have demonstrated that IL-13 possesses several unique effector functions that distinguish it from IL-4 (Wynn, 2003). These functions include a variety of biological activities, such as tissue eosinophilia and mastocytosis and the prolongation of eosinophil survival through inducing autocrine production of IL-3, IL-5, and GM-CSF (SchmidGrendelmeier *et al.*, 2002, Shakoory *et al.,* 2004, Wynn, 2003). Eosinophils notably expresses functional IL-13 in eosinophilic inflammatory diseases. IL-13 upregulates the vascular cell adhesion molecule (VCAM) on endothelial cells and stimulates epithelial cells to produce eotaxin (Rankin *et al.,* 2000, Stone *et al.*, 2010). Notably, allergic inflammation is driven by Th2 cells (Galli *et al.,* 2008). Th2 cells produce IL-4, IL-5 and IL-13 (Vijayanand *et al.,* 2012, Zhu, 2015).

The dominant cytokines produced by leukocytes differ; basophils express abundant IL-4 and IL-13 but little IL-5, whereas mast cells produce IL-5 and IL-13 and less IL-4 (Stone *et al.,* 2010). Furthermore, it has been proposed that the effect of IL-13 might be chemokinedependent and a potent inducer of eosinophil-active chemokines, including eotaxin and Rantes (Pope *et al.,* 2001). However, IL-13 also directly stimulates eosinophil activation, chemoattraction, and their survival *in vitro* (Park and Bochner, 2010, Schmid-Grendelmeier *et al.,* 2002).

Stem cell factor (SCF)/c-kit ligand is a critical growth factor and chemoattractant for mast cells which also regulates their activation, degranulation, survival and chemotaxis (da Silva *et al.,* 2014). Fibroblasts as well as keratinocytes and endothelial cells secrete SCF (Welker *et al.,* 1999), but other cells such as eosinophils and mast cells may also serve as a source of SCF (da Silva *et al.,* 2006).

T cells and macrophages are the main cells which produce TGF-β (Link and Xiao, 1998), but other cells including mast cells and eosinophils also do so (Rich *et al.,*

2012). TGF-β antagonises the effect of IL-5 on eosinophils (Luttmann *et al.*, 1998, Xie Q, 2011). It also plays an important role in the deposition of extracellular matrix proteins during chronic inflammation and fibrosis, and modulates wound healing as well as tissue remodelling (Wynn, 2008).

IFN-γ is the main cytokine of T cells and, among other functions, it is responsible for macrophage activation (Desport, 2010, Schoenborn and Wilson, 2007). It also inhibits eotaxin (Miyamasu *et al.*, 1999, Sato *et al.*, 2004).

1.12 Overview of advanced diagnostic techniques applied in human and veterinary medicine

Alternative advanced and diagnostic molecular tools provide excellent means to visualise transcript cellular molecules such as those produced by gene expression (Kukurba and Montgomery, 2015). Nowadays, a number of molecular tools are used for diagnostic assays and research purposes as they provide better quantitative results, and improved visualisation of more than a target in a single tissue and allow the demonstration of biomarkers (Cassidy and Jones, 2014). Immuno-histochemistry (IHC) and *in situ* hybridisation (ISH) techniques are vital diagnostic tools to visualise disease-causing pathogens and to characterise the associated morphological changes that occur within the tissues (Maes *et al.*, 2014).

IHC is a vital diagnostic and research tool used to recognise specific proteins of pathogens *in situ* and detect, localise and quantify other antigens in fixed tissues with preserved morphology (Carvajal-Hausdorf *et al.*, 2015, de Matos *et al.*, 2010, McRae *et al.*, 2009, Stack *et al.*, 2014). ISH, on the other hand, is a molecular analysis method that allows *in situ* detection of nucleic acids in tissue sections, applicable to identify DNA, mRNA and microRNAs in cells (Jensen, 2014, Maes *et al.*, 2014, Wang *et al.*, 2012). Like PCR, ISH is used to identify and recognise a specific complementary sequence of nucleic acids of pathogens and markers within tissues (Jensen, 2014).

Concurrently, advanced RNA-ISH approaches have been developed which use hybridisation with a signal amplification system to increase the sensitivity of the technique (Maes *et al.*, 2014, Resende *et al.*, 2017, Wang *et al.*, 2012). This technique targets sets of up to 20 oligonucleotide pairs and aims to detect individual

mRNA molecules with single cell resolution and ultimately enables their visualisation through chromogenic and fluorescent applications. This method allows for considerable amplification of the signal and provides high sensitivity while reducing the non-specific binding of the preamplifier DNA; thereby reducing nonspecific background staining (Wang *et al.,* 2012). This RNA-ISH technique has been applied successfully to diagnosis and research in human and animal medicine; especiallywith regard to the diagnosis of infectious diseases (Chang *et al.,* 2015, Gaynor *et al.,* 2016, Joshi *et al.,* 2016, Luff *et al.,* 2016, Palinski *et al.,* 2016, Palmer *et al.,* 2015).

1.13 Aims and objectives

Eosinophilic granuloma (EG) is a chronic inflammatory skin reaction in horses that is characterised by intensive infiltrates of eosinophils, macrophages as well as lymphocytes and plasma cells. In contrast, cutaneous mast cell tumours (MCT) are neoplastic processes that arise from neoplastic mast cells and their progenitors. In contrast to EG, MCT are rare in horses. Both conditions show overlapping histological features in horses. This can lead to misdiagnosis and confusion.

The aim of this thesis is to thoroughly assess the histopathological features of EG and MCT in the skin so as to distinguish between the two lesions and enable the defining and description of morphological criteria for MCT and EG. A further aim is to investigate the interaction between inflammatory cells (eosinophils, macrophages, mast cells and lymphocytes) in equine EG and MCT. This may lead to a furtherance of existing understanding of the pathogenic mechanisms of the two lesions and the possible interactions that exist between them. In this study, a total of 191 lesions comprising equine eosinophilic granulomas and mast cell tumours were investigated. Histological, histochemical, immunohistochemical and molecular (RNA-ISH) investigations were undertaken.

CHAPTER 2: MATERIALS AND METHODS

2. Materials and methods

2.1. Source and origin of case material

A total of 191 archived, formalin-fixed, paraffin embedded (FFPE) samples of MCT (n=62) and EG (n=129) from a total of 153 horses were re-examined for this project. Samples were taken from cases admitted and/or referred to the University of Liverpool Philip Leverhulme Equine Hospital and referral equine local veterinary practices between 2005 and 2015. Lesions were originally diagnosed as MCT or EG by the veterinary pathologists involved in the Veterinary Laboratory Services, Institute of Veterinary Science, University of Liverpool (VLS), based on their histological features.

2.2 Collection of clinical data

Signalment and clinical information of the affected horses were collected from archived data from the VLS database as follows: age (years), breed, sex, neutering status, size of lesion (major axis; cm), single or multiple lesion, primary or recurrent lesion, and initial diagnosis (MCT or EG).

2.3 Histological examination

2.3.1 Re-examination of archived diagnostic cases

All cases were histologically re-assessed, using haematoxylin-eosin (HE) and toluidine blue stained archived sections, based on typical morphological and histological criteria (see 2.4). When the old slides exhibited insufficient staining quality (fading due to age and so on) and for more detailed examinations, new sections were prepared from the paraffin blocks and stained with HE (see 2.3.3).

2.3.2 Preparation of tissue sections

From all cases, consecutive sections (4 µm thickness) were prepared from paraffin blocks using a Leica RM2125 RTS microtome and mounted on microscopic slides. After drying at 37ºC for 30 minutes (for the full Histology Standard Operating Procedure, see Appendix I), sections were stained with HE and toluidine blue (TB)

(section 2.5.1.) Further sections were subjected to special stains, and for immunohistochemical staining (IHC) and mRNA-*in situ* hybridisation (mRNA-ISH). For IHC, sections were mounted on poly Lysine treated slides (Solmedia, Shrewsbury, UK) and for RNA-ISH staining, they were mounted on SuperFrost® Plus slides (Thermo Scientific, Braunschweig, Germany).

2.3.3 Haematoxylin-eosin staining

The HE stain was performed by the Histology Laboratory, VLS, as a routine stain and according to its world-wide use in histopathological examinations (Fischer *et al.*, 2008, Cardiff *et al.*, 2014). The HE stain highlights cells' nuclear structures in blue and the cytoplasm as well as many extra-cellular components in various shades of bright pink. The structures do not have to be acidic or basic to be called basophilic and eosinophilic; this terminology is based on the affinity of the structures and material to the dyes (Suvarna *et al.*, 2012). Briefly, consecutive paraffin sections (4 µm thickness) were deparaffinised in xylene twice for 5 minutes each and rehydrated in descending grades of alcohol (100%, 96%, 85%, and 70%) to distilled water. Sections were then incubated in Mayer's Haematoxylin (see Appendix 1) for 5 minutes, blued in running tap water for 6 minutes, and stained in eosin for 2 minutes. This was followed by quick dehydration through 96% alcohol for 3 times for 1 minute each (dip sections up and down to remove excess eosin) and 3 changes of 100% alcohol. After clearing in xylene, (2 changes, 3 minutes each), cover slips were mounted on the slides with DPX mounting medium (Thermo Scientific, LAMB/DPX).

2.4 Categorisation and grouping criteria

All cases (HE-stained sections) were histologically examined independently by the candidate (AM) and one supervisor (LR) who were both blinded to the initial histopathological diagnosis. The histological features were initially assessed in accordance with previously published work that describes equine EG and MCT (Goldschmidt and Hendrick, 2002, Maxie, 2007, Scott and Miller, 2011b, Scott and Miller, 2011c, Zachary and McGavin, 2012).

Upon re-evaluation of the lesions, a diagnosis of EG was made based on the following criteria: nodular to diffuse focal infiltrates of eosinophils in association

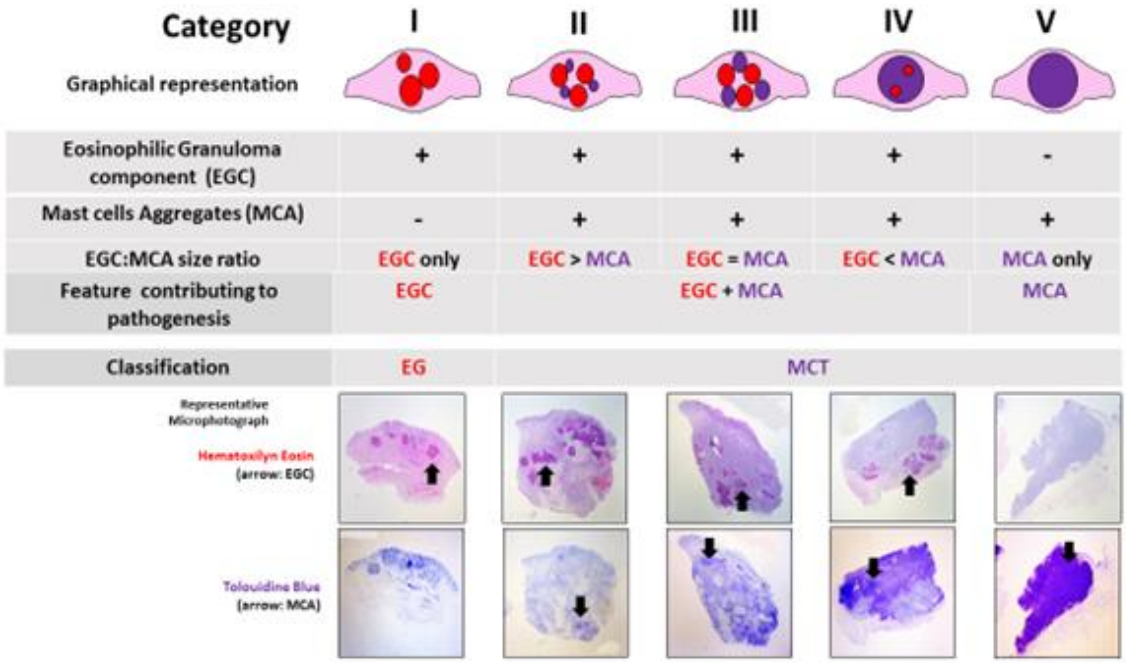
with foci of collagen degeneration (flame figures) which were occasionally mineralised and surrounded by a granulomatous inflammation that sometimes formed a palisading granuloma with a centre composed of eosinophilic material (interpreted as degenerated eosinophils). This was variably associated with lymphoid nodules, with or without small aggregates of or individual mast cells.

Lesions were classified as MCT according to the following criteria: Presence of large monomorphic aggregates of well-differentiated or poorly differentiated neoplastic mast cells, referred to as "mast cell aggregates" (MCA), often involving the dermis and subcutis and variably associated with variable numbers of mature eosinophils, and with focal to multifocal areas of EG.

When lesions did not fulfil the above criteria and differed substantially from a diagnosis of EG or MCT, the cases were classified as new morphological entities. These were excluded from further analysis and described separately (see Appendix II-section B) as 4 cases with histiocytic morphological.

Based on the histological findings, the cases were classified and grouped into five categories (Figure 1). A subpopulation of cases (n=95) was then selected (see 3.4) and subjected to further IHC examinations (see 3.5). Based on the staining quality and results of the latter, a total of 10 cases were then selected for the RNA-ISH study (see 3.6).

Figure 1: Overview of the different categories, groups and diagnoses (classification), based on the presence and relative abundance of EGC and MCA



2.5 Special stains

A set of histochemical stains was used to highlight different features within the lesions.

These included Toluidine blue stain, Lendrum's stain (Carbol Chromotrope), Von Kossa stain, Masson Trichrome, and Perl's Prussian blue stain (PERLS). The stains were prepared by the technical staff of the VLS Histology Laboratory.

2.5.1 Toluidine blue (TB) stain for the demonstration of mast cell granules

As a common and widely used histochemical stain, Toluidine blue (TB) stain was used to identify mast cells through demonstration of their metachromatic granules, (Bancroft and Gamble, 2008). Briefly, sections (4 µm) were deparaffinised and hydrated in distilled water. Slides were placed in TB working solution for 2-3 minutes and washed three times in distilled water. This was followed by quick dehydration through 95% and twice 100% alcohol (10 dips each, since stain fades quickly in alcohol), and clearing in xylene, (2 changes, 3 minutes each). Cover slips

were mounted on slides with DPX. Upon bright field light observation, metachromatic stain (purple) was considered indicative of mast cell granules.

2.5.2 Lendrum's stain (Carbol Chromotrope) for demonstration of eosinophil granules

Lendrum's stain was applied, as it selectively and vividly stains the granules of eosinophils thereby confirming the presence of eosinophils (Lendrum, 1944). Briefly, sections were initially deparaffinised in xylene for 5 minutes and rehydrated through graded alcohol, then incubated in Mayer's Haematoxylin (see Appendix 1) for 1 minute and blued in running tap water for 5 minutes. Sections were then stained in carbol chromotrope (Clin-Tech Ltd, 641550; Guildford, UK) for 1 h (see Appendix I for Solutions) followed by rinsing in distilled water. This was followed by dehydration with ethanol and clearing with xylene.

Cover slips were mounted on slides with DPX. An intense red granular stain highlighted the granules of eosinophils, and a blue stain was indicative of nuclei.

2.5.3 Von Kossa stain for confirmation of mineralisation

The Von Kossa (calcium) stain was performed to confirm the presence of mineralisation, as it is used for the histological visualisation of calcium deposits in FFPE or frozen sections (Bancroft and Gamble, 2008). It also helps to accurately characterise the morphological features of localised mineral deposition in inflammatory foci (granulomas), degenerative tissue, and necrosis (Mauldin and Peters-Kennedy, 2016). Briefly, sections were deparaffinised in xylene for 5 minutes and rehydrated through graded alcohol and distilled water. They were then incubated in 5% sliver nitrate and exposed to strong light (500-watt floodlight attached to a retort stand about 0.5 m above the sections) for 15 minutes, followed by washing in distilled water (3 changes) and counterstaining in 1% neutral red (see Appendix 1for solutions) for 1 minute. This was followed by washing in distilled water and blotting with fibre free paper and quick dehydration with ethanol and clearing with xylene. Cover slips were mounted on the slides with DPX. A dark black stain confirmed calcium salt deposition (mineralisation).

2.5.4 Masson trichrome stain to demonstrate collagen fibres

The Masson trichrome stain was applied to demonstrate collagen fibres in EG areas, as a common and widely used histochemical stain to visualise collagen fibres FFPE tissues (Bancroft and Gamble, 2008). Briefly, sections were deparaffinised in xylene for 5 minutes and rehydrated through each graded alcohol with distilled water, then subjected to Celestine blue stain for 5 minutes. This was followed by rinsing with tap water and staining with Mayer's Haematoxylin (see Appendix 1) for 5 minutes and blueing in tap water for 6 minutes. Sections were then subjected to cytoplasmic stain (see Appendix 1) for 7 minutes, rinsed with water and differentiated in 1% phosphomolybdic acid solution for 5 minutes and again rinsed with water. This was followed by counterstaining in 2% light green solution (see Appendix I) for 5 minutes, and differentiation in 1% acetic acid solution for 3-5 minutes to remove excess green, and blotting with fibre-free blotting paper. Sections were then dehydrated rapidly in absolute alcohol, cleared, and cover slips were mounted with DPX. Collagen fibres were stained dark green, nuclei black, and the background appeared red.

2.5.5 Perls' Prussian blue stain for the demonstration of hemosiderin

The Perls' Prussian blue was performed to detect iron within macrophages (siderophages). Hemosiderin, is found in macrophages and degraded haemoglobin (Bancroft and Gamble, 2008). Briefly, after deparaffinisation in xylene for 5 minutes and rehydration through graded alcohol and distilled water, sections were placed in plastic slide holders and flooded with a mixture of Ferro cyanide and hydrochloric acid (see Appendix I) for 30 minutes, followed by rinsing in running tap water for 5 minutes. Sections were counterstained with filtered neutral red stain for 1 minute (see Appendix I) and rinsed with distilled water. They were then dehydrated rapidly in absolute alcohol, cleared, and cover slips were mounted with DPX. Haemosiderin appeared as deep blue colour and identified macrophages with iron (hemosiderin), i.e. siderophages, within the lesions.

2.6 Morphological parameters

EG and MCT lesions were evaluated to assess a set of morphological criteria. For this purpose, a specific nomenclature was defined to characterise different areas in the sections:

- "**Lesion**": the area occupied by the pathological entity regardless of its classification
- "**EG**": a lesion diagnosed as eosinophilic granuloma
- "**MCT**": a lesion that is in its entirety diagnosed as mast cell tumour
- "**MCA**": aggregates of ≥5 mast cells forming clusters. These are present in both EG and MCT.
- "**EGC**": an eosinophilic granuloma component, composed of a core of eosinophils, degranulated and degenerated with and /or without collagen.

Furthermore, after initial screening, a range of morphological features was observed. Subsequently, all lesions were evaluated systematically for their presence, extent, and distribution. These were as follows:

- EGC and MCA within EG and MCT
- Mitotic index (MI)
- Density of viable eosinophils in mast cells aggregates (MCA)
- Lymphocyte aggregates (presence and number of lymphocyte aggregates (nodules) within lesions)
- Location of the lesion
- Ulceration
- Association of EGC with hair follicles
- Mineralisation
- Presence of collagen fibres within areas of EGC
- Presence of individual mast cells (not forming aggregates)

2.6.1 EGC and MCA within EG and MCT

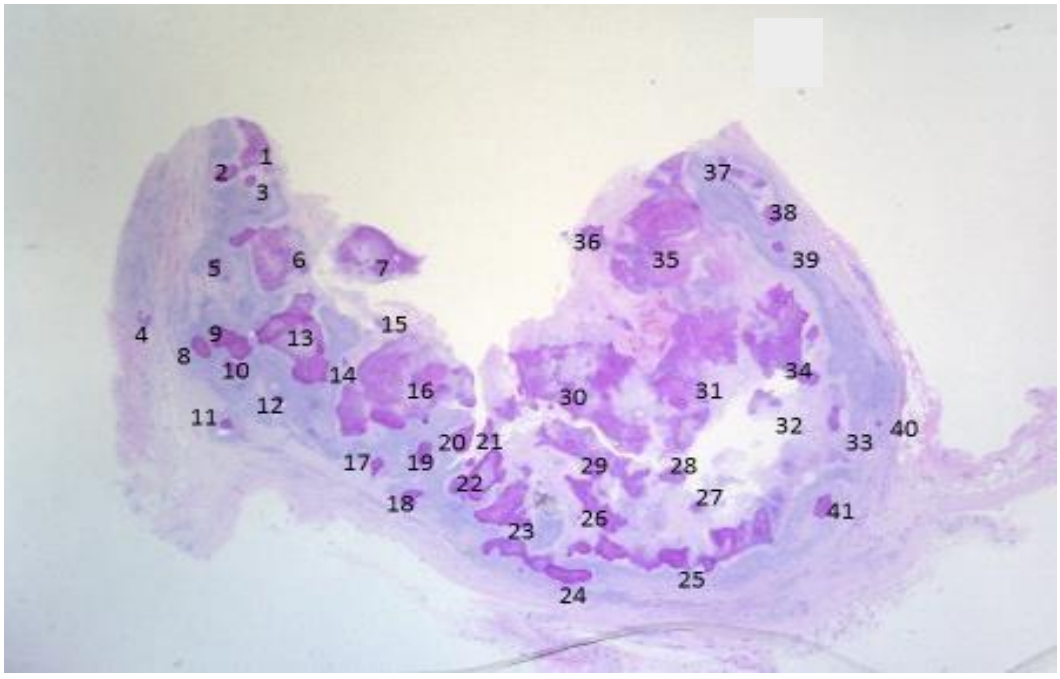
Briefly, the areas occupied by EG and MCA were measured in all lesions. First a low magnification digital scan (Slides 2 PC Express scanner; ION, Cumberland, USA) was acquired using an imaging software program (Media2 Arcsoft, Freemont

USA), then a low magnification digital picture was taken (captured) from all HE-stained sections.

Secondly, the low magnification digital picture (which was captured earlier) was further analysed by using an image analysis software program (Image JI.48V/JAVAL.6.0-20 (64bit) to obtain the area for each lesion; the size of the entire occupied area was measured as well as the area occupied by MCA and /or EGC.

For cases with more than one section from the lesion, the most representative section was chosen. In lesions with more than one area of EGC and/or MCA, the sum of all areas was taken for each type of change. The same picture was labelled using Microsoft PowerPoint. A representative picture of the assessment is presented in Figure 2.

Figure 2: Representative of a scanned picture of equine eosinophilic granuloma lesion stained by HE stain



This shows the labelled area (using Microsoft PowerPoint, as captured by the Ion.com software program) to be measured by software (Image JI.48V/JAVAL.6.020 (64-bit). Arabic numbers denote areas matched by colour and numbered for the purpose of analysis.

2.6.2 Mitotic index (MI)

In MCA, the mitotic index (MI) was assessed in HE-stained sections, using a light microscope (Leica DM500; Leica Microsystems (Schweiz) AG, Heerbrugg, Switzerland). Thereafter by counting the number of mast cells exhibiting mitotic figures in 10 high power fields (HPF, x 400).

2.6.3 Proportion of viable eosinophils in mast cells aggregates (MCA)

The proportion of viable eosinophils within MCA was assessed in HE-stained sections by using the light microscope with high magnification (x 400), using a semi-quantitative scoring system ranging from 0-100% of the area occupied by the cells. This was done as a percentage (%) of the area occupied by viable eosinophils in 10 HPFs, and was thus not likely to have been affected by the "total number" of eosinophils. In lesions with more than one area, the sum of the scores was taken, then the average was calculated.

2.6.4 Lymphocyte aggregates

All lesions were assessed in HE-stained sections for the presence of lymphocyte aggregates, using the light microscope at x 200 magnification, and by counting the total number of lymphocyte aggregates for each lesion.

2.6.5 Location of the lesion

The location of the lesion within the dermis and subcutis was assessed in HE-stained sections, using a light microscope at x 200 magnification. A "scoring system" ranging from 1 to 4 according to the depth of lesion was applied.

- Superficial dermis: the area of the dermis extending from the dermo-epidermal junction to the level of the sebaceous glands [score 1];
- Mid dermis: the area stretching from the sebaceous glands to the base of the hair bulb [score 2];
- Deep dermis: the area below the hair follicle structures down to the subcutis [score 3];
- Subcutis: the adipose tissue below the dermis [score 4].

When the lesion stretched over more than one area, scores for each area were given, and the average was used to express a "depth score".

2.6.6 Ulceration

Lesions were assessed in HE-stained sections for the presence of ulceration with the light microscope on HE slides. For scoring purposes, 0 indicated an absence of ulceration and 1 indicated its presence.

2.6.7 Association of EGC with hair follicles

Lesions were examined in HE-stained sections for the presence of EGC in spatial association with hair follicles (i.e. EGC found in anatomical contact with any part of a hair follicle). For scoring purpose, 0 indicated an absence of EGC with hair follicles and 1 indicated its presence.

2.6.8 Mineralisation

Lesions were examined for the presence of focal mineralisation. This was initially evaluated in HE-stained slides, where it appeared as areas of granular basophilic amorphous material. This was later confirmed with the Von Kossa-stained sections. For scoring purposes, 0 indicated the absence of mineralisation and 1 indicated its presence.

2.6.9 Presence of collagen fibres within areas of EGC

Lesions were examined for the presence of collagen fibres within areas of EGC (represented by eosinophilic areas with cellular debris and bright fibrillar material (Scott and Miller, 2011b). Later this finding was further investigated with Masson's Trichrome stain: collagen fibres were considered to be present when fibrillar bright green material was observed within the core of EGC, using a high magnification (x 400). For scoring purposes, 0 indicated the absence, and 1 the presence, of collagen fibres within areas of EGC.

2.6.10 Presence of individual mast cells

The presence of individual mast cells independent of MCA was assessed in TB-stained sections, using a light microscope at high magnification (x 400). For scoring purposes, 0 indicated the absence, and 1 the presence, of individual mast cells in EG (equine eosinophilic granuloma) lesions only.

2.6.11 Evaluation of different colours after Lendrum's stain in the core of EG

In Lendrum's-stained sections, the granules of viable eosinophils were found to be bright red, as expected. However, different colours (purple to bright red areas of EGC together with pale red and colourless areas) representing degranulated or non-viable eosinophils were observed in EGC areas.

To assess the colour for each lesion the procedures were as follows: the areas occupied by EGC were measured in all lesions, first by using a low magnification digital scan (Slides 2 PC Express scanner; ION, Cumberland, USA) acquired with the Ion software program (Media2 Arcsoft, Freemont USA), then a low magnification digital picture was taken (captured) from all Lendrum's-stained sections.

Secondly, the low magnification digital picture (which was captured earlier) was further analysed using an image analysis software program (Image JI.48V/JAVAL.6.0-20 (64bit) to obtain the area for each single EGC within the lesion.

Thirdly, the colour was assessed for each single EGC (which was measured earlier) based on the colour intensity (namely; purple, intense red, pale red and colourless) using a light microscope at x 200 magnification. Asemi-quantitative scoring system was adopted ranging from 0-100%. (i.e. for each single area of EGC represented as 100% in total, the different colours were assessed semi-quantitatively. Area representative for each colour was estimated as a fraction of the total EGC area and expressed as percentage. This is further explained at 3.4.12.

2.7 Immunohistochemistry (IHC)

Immunohistochemistry (IHC) is a methodology widely used in diagnostics and research into veterinary pathology (Ramos-Vara, 2005). To investigate the different inflammatory cell populations involved in EG and MCT a set of immune-histochemical stains was applied to identify relevant immune cells. These included: T cells (CD3-positive/CD79a1negative/Iba-1-negative), B cells (CD79a1-positive/ CD3 negative/Iba1-negative), and macrophages (Iba1-positive/CD3-negative/ CD79a1-negative). Other markers included proliferating cell nuclear antigen

(PCNA) to investigate cell proliferation, CD117 (c-Kit) for mast cells, mast cell tryptase for mast cell degranulation, lysozyme for tissue macrophages, and RANTES (CCL5) for T cells and macrophages. All stains were performed by the technical staff of the VLS Histology Laboratories apart from the staining for RANTES (CCL5) which was performed at the Histology Laboratory, Institute of Veterinary Pathology, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland. Details of the antibodies used and the cell population to which they are directed are shown in Table 2.

Table 2: Antibodies used for immunohistological staining

Ligand	**Antibody (incl. clone) and sources**	**Specificity and references (horse)**
CD3	Rabbit anti human CD3, DAKO A0452, Glosturp, Denmark (NI589)	Pan-T cell marker (Collins Kelley *et al.*, 1997, Delcambre *et al.*, 2016, Kappe *et al.*, 2009)
CD79a	Mouse anti-human CD79 (AbD serotec, MCA2538H)	Pan-B cell marker (Hinden *et al.*, 2012, Jones *et al.*, 1993, Lee *et al.*, 2013)
Iba-1	Primary antibody Iba1 goat Anti-AIF1 (LifeSpan BioSciences Inc, LS-B2402)	Pan-macrophage marker (Delcambre *et al.*, 2016)
Proliferating cell nuclear antigen (PCNA)*	Mouse anti-PCNA, clone PC10, (DAKO, M0879)	Proliferating cells in S phase, late G1 and early G2 phase (Kubben *et al.*, 1994, Ressel *et al.*, 2015)
CD117 (c-Kit)*	Rabbit anti-human CD117, ckit (DAKO, A4502)	Neoplastic mast cells (Ressel *et al.*, 2015)
Mast cell tryptase*	Mouse anti-human mast cell tryptase, clone AA1, (Dako, M7052)	Mast cells (Clements *et al.*, 2012, Hinden *et al.*, 2012, Pickles *et al.*, 2010)
Lysozyme*	Rabbit anti-human lysozyme (Dako, A0099)	Tissue histiocytes (macrophages) (Kappe *et al.*, 2009)
RANTES (CCL5)	Polyclonal rabbit antihuman RANTES (Abcam, ab83324): Cross-reacts with horse (90% homology with the equivalent horse peptide).	T cells and macrophages (secreted).

*****These antibodies were only used for the characterisation of the four tumours with unique features described in the manuscript in Appendix II, section B but excluded from the main study.

2.7.1 IHC staining protocols

For all IHC staining, paraffin sections (4 µm) were prepared and mounted on Poly Lysine Coated Adhesive White Frosted Clipped Microslides (Solmedia, MSS61012S), except for RANTES which was mounted on SuperFrost® Plus slides (Thermo Scientific). The autostainer protocol (Agilent Technologies Ltd, Stockport, UK),was used for CD3, CD79a, Iba1, CD117(c-Kit), Mast cell tryptase and Lysozyme. The manual protocol was used for PCNA and RANTES.

2.7.1.1 Deparaffinisation

Sections were dewaxed in xylene (2 x 5 minutes), and rehydrated through graded alcohol: 100% ethanol (2 x 5 minutes), 96% ethanol (1 minute), 80% ethanol (1 minute), 70% ethanol (1 minute), and distilled water (1 minute).

2.7.1.2 Antigen retrieval

When the autostainer was used, antigen retrieval was undertaken in Dako PT buffer high pH or low pH (Agilent Technologies Ltd, Stockport, UK), using a computer-controlled antigen retrieval workstation (PT Link; Agilent Technologies Ltd) and a 20 minutes incubation at 98°C. For the manual protocols, antigen retrieval was undertaken with citrate buffer pH 4 at 96ºC for 30 minutes; for RANTES (CCL5) sections were incubated in EDTA solution in a Pascal pressure cooker (Dako, Glostrup, Denmark) at 97ºC for 20 minutes.

A summary of the antigen retrieval methods is given in Table 3.

Table 3: Antigen retrieval methods

Ligands	Target retrieval solution
CD3	High pH citrate buffer, pH 9.0 (Dako, K8004)
CD79a	Low pH citrate buffer, pH 6.1(Dako, K8005)
Iba1	High pH citrate buffer, pH 9.0 (Dako, K8004)
Proliferating cell nuclear antigen (PCNA)*	Citrate buffer, pH 4.0 (Dako, PC10)
CD117 (c-Kit)*	High pH citrate buffer, pH 9.0 (Dako, K8004)
Mast cell tryptase*	Low pH citrate buffer, pH 6.1(Dako, K8005)
Lysozyme *	High pH citrate buffer, pH 9.0(Dako, K8004)
RANTES (CCL5)	High pH (EDTA buffer, pH 9.0; see Appendix I)

*These antibodies were only used for the characterisation of the four tumours with unique features described in the manuscript in Appendix II, section B but excluded from the main study.

2.7.1.3 Blocking of endogenous enzymes

When the autostainer was used, blocking of endogenous enzymes was undertaken in Dako (Agilent Technologies Ltd, Stockport, UK), by using EnVisionTM FLEX Peroxidase-Blocking Reagent (SM801) for (CD3, CD79a, Iba1, CD117(c-kit), Mast cell tryptase and Lysozyme. For RANTES (CCL5) slides were washed in distilled water, assembled in Shandon Sequenza racks (Thermo Fisher Scientific) with Wash Buffer (Dako, S3006), incubated with Peroxidase-Blocking Solution (Dako, S2023) for 10 minutes at room temperature (RT), and washed with Wash Buffer for 10 minutes. For PCNA, slides were placed in cover plates in Sequenza racks and washed 3 times each for 2 minutes with Tris Buffered Saline (TSBT) (TBST + 0.05% Tween 20) (see AppendixI) followed by blocking with Peroxidase-Blocking Solution (Dako, S2023) for 10 minutes at RT.

2.7.1.4 Antibody labelling and visualisation

When an automated immunostainer was used (Link 48; Agilent Technologies Ltd), slides were then incubated with the primary antibodies, against CD3 (polyclonal rabbit anti-human CD3, Agilent Technologies Ltd (Dako A0452); 1:500), CD79a (monoclonal mouse anti-human CD79a, AbD Serotec (MCA2538H); 1:300), Iba-1 (Anti-AIF1/IBA1; Bioscience (LSB2402); 1:500), KIT protein (rabbit anti-human CD117 [c-Kit], Agilent Technologies Ltd. [A4502]; 1: 500), lysozyme (rabbit anti-human lysozyme, Agilent Technologies Ltd. [A0099]) and mast cell tryptase (mouse anti-human MCT, clone AA1, Agilent Technologies Ltd.; 1: 500); after blocking with 2.5% horse serum for 10 minutes. All antibodies were diluted in EnVisionTM FLEX Antibody Diluent (Dako, K8006).

Slides were incubated for 20 minutes at RT. This was followed by a 20 minute incubation at RT with the secondary antibody and polymer peroxidase-based detection system (Anti Mouse/Rabbit Envision Flex+, Dako) and for Iba1, the secondary reagent was anti-goat IgG (ImmPRESS™ HRP Reagent Kit Peroxidase; MP-7405). The reaction was visualised with 3,3′ diaminobenzidine (Agilent Technologies Ltd).

For RANTES (CCL5), sections were incubated overnight at 4ºC with a rabbit polyclonal anti-RANTES antibody (ab83324, Abcam) diluted 1:300 in antibody diluents (Dako, S0809). After primary incubation, slides were washed in wash buffer (Dako, S3006) for 10 minutes and incubated with EnVision-HRP anti-rabbit polymer (Dako, K4003) for 30 minutes at RT, then washed in wash buffer for 10 minutes, removed from the Sequenza racks and put into distilled water. Then, slides were incubated with a DAB chromogen solution (Dako, K3468). For PCNA, sections were incubated overnight at 4ºC with the primary antibody diluted 1:100 in TBST. Slides were then washed in TBST 3 times for 2 minutes each, then incubated for 30 minutes in EnVison-HRP anti-mouse polymer (Dako, K4001). After washing in TBST 3 times each for 2 minutes, slides were removed from the Sequenza clips and washed three times with distilled water. Slides were then incubated for 10 minutes in 3,3’ diaminobencidintetrahadrochloride (DAB, Fluka Chemie AG, Buchs, Switzerland) with 0.01% H_2O_2 (Perhydrol 30% H2O2P-a;

Fisher Scientific) in 0.1M imidazole buffer (pH7.2; Fluka Chemic AG; see Appendix I) at RT and washed in distilled water three times for 5 minutes each.

2.7.1.5 Counterstaining

For RANTES (CCL5), the slides were washed in wash buffer for 10 minutes and counterstained with Gill's Haematoxylin II (Merck, 105175). For PCNA, the slides were counterstained with Papanicolaou's heamatoxlin (Merck 1.09254.0500) for 1 minute, then placed in running tap water for 5 minutes. All the other markers slides were counterstained with an autostainer using EnVisionTM FLEX Hematoxylin (Dako, K8008).

2.7.1.6 Dehydration, clearing and mounting

When the autostainer was used, slides were removed from the stainer and dehydrated through graded alcohols (96% ethanol for 1 minutes, 100% ethanol twice for 3 minutes), cleared in xylene (2 min, 2 x 3 min), and cover slipped with Distyeren Plasticiser Xylene (DPX, BDH brand, VWR International).

For RANTES (CCL5), slides were dehydrated in ascending ethanol (70% ethanol for 1 minute; 95% ethanol for 1 minute; 100% ethanol twice for 2 minutes each), cleared in xylene (twice for 2 minutes each) and mounted with cover slips and xylene-based mounting medium. For PCNA, sections were dehydrated in ascending ethanol (96% for 1 minutes, 100% for 2 minutes and 3 minutes), cleared in xylene (for 2 minutes and twice 3 minutes), and cover slipped with DPX.

2.7.1.7 Positive tissue controls

Equine skin with normal mast cells and a lymph node served as positive controls for KIT, and Mast Cell Tryptase, respectively. Equine lymph node served as a control for CD3, CD79a, lysozyme, PCNA and Iba1. A positive control was run in all the experiments.

2.7.1.8 Negative controls

Consecutive sections incubated with non-immune rabbit serum or a murine subclassmatched unrelated monoclonal antibody served as negative controls. A negative control was run in all the experiments.

2.7.1.9 Determination of positive reactions and protein expression in tissue sections

A distinct brown colour was considered indicative of a positive reaction if cytoplasmic (KIT, Iba-1, Mast cell tryptase, lysozyme, PCNA in mitotic cells), membranous (KIT) or nuclear (PCNA). The KIT expression pattern was determined according to previously described parameters (Ressel *et al.,* 2015), where a membranous staining reaction is considered as normal, and a cytoplasmic, focally stippled or diffuse staining as aberrant.

2.7.1.10 Quantitative assessment of immunohistochemical staining

For T cells (CD3+), B cells (CD79a+) and macrophages (Iba-1+), cell numbers were estimated semi-quantitatively at 200 x magnification in 10 HPF selected areas of the sections. The semi-quantitative scoring system was adopted ranging from 0-100% (i.e. the total area represent as 100%, then the distributed positive cells were estimated in percentage related to the total area). In addition, an overall evaluation was performed at low power estimating the area of the section that was occupied by the respective cells.

For RANTES (CCL5), the following scoring system was adopted by Dr Fernando RomeroPalomo as part of his postdoctoral project at the Institute of Veterinary Pathology, Vetsuisse Faculty, University of Zurich: the mean count of positive cells was determined in 25 fields of 0.2 mm^2 (x400) and scored as follows: Score 0 (0 cells), 1 (1-10 cells), 2 (11-20), 3 (21-40), 4 (41-50).

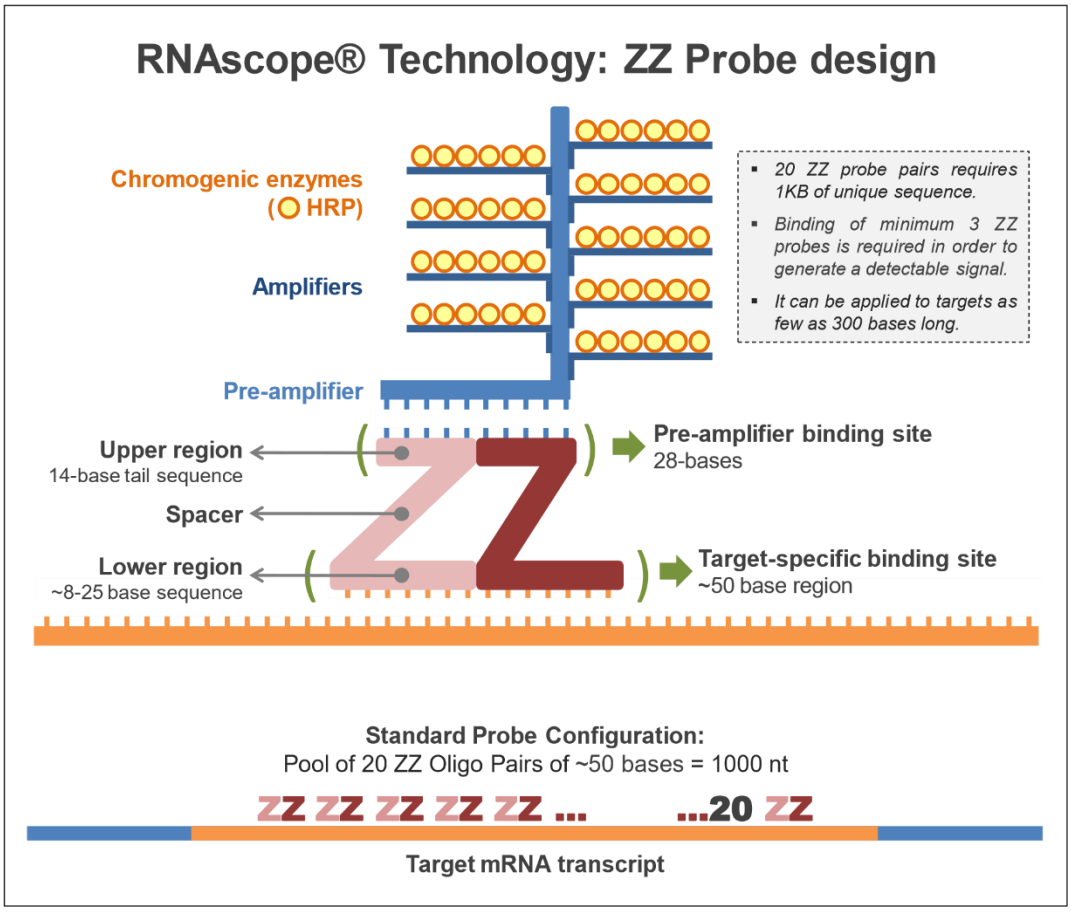
2.8 Statistical analysis

The statistical association between morphological parameters was investigated with the chi-square (for category variables) or Mann-Whitney U (for numerical variables) statistical tests, using the SPSS 13 Software (SPSS 13.0, SPSS Inc., IBM Chicago, Illinois, USA). Correlation between IHC marker (CD3, C79a and Iba1) and cytokine (IL5, IL13, IL4, CCl1, MIP1a and CCL5) scores was determined using Pearson's Rho (R). Statistical significance was set at 5% (0.05) and 1% (0.01).

2.9 mRNA-in situ hybridisation (mRNA-ISH)

RNA- ISH, a technique used to identify the location of mRNAs in fixed tissue sections with the help of anti-sense RNA probes specific for the mRNA target (Grabinski *et al.*, 2015, Jensen, 2014), was used to obtain further information on the interaction of the involved immune cells (i.e. eosinophils, macrophages, lymphocytes and mast cells). RNA-ISH was performed by Dr. Fernando Romero-Palomo at Institute of Veterinary Pathology, University of Zurich. The RNAscope® technology (Advance Cell Diagnostics - ACD Inc., USA) was applied based on the technology previously described by Wang *et al.,* (2012) as shown in Figure 3.

Figure 3: RNAScope technology overview



Source: Fernando Romero-Palomo

RNA-ISH was performed on selected cases (see Appendix 2-section A) and comprised the following markers: MIP1α/CCL3, eotaxin/CCL11, interleukin 4 (IL-4), interleukin 5 (IL-5), Stem Cell Factor (SCF) (aka cKIT ligand), transforming growth factor beta (TGF-β), and interleukin-13 (IL-13) (Table 4); the respective mRNA sequences were taken from the NCBI website (Table 5).

Table 4: List of cytokines for which an RNA-ISH was established

Cytokines/ Interleukin	Mainly cell producers
CCL11 / Eotaxin	Mainly secreted by epithelial cells macrophages, T cells, eosinophils, mast cells
MIP1α/CCL3	Mainly secreted by epithelial cells, MPH, T cells, EOs, MCs
Interleukin 4 (IL-4)	Mainly secreted by T cells and MCs (also described by EOs).
Interleukin 5 (IL-5)	Mainly secreted by T cells and MCs
Interleukin 13 (IL13)	Mainly secreted by T cells
Stem cell factor (SCF)/c-kit ligand	c-kit ligand: Mainly secreted by fibroblast, EOs and MCs
Transforming growth factor beta (TGF-β)	Mainly secreted by T cells and MPH (also EOs, MCs…)

Table 5: Probes and mRNA sequences of the selected chemokines/cytokines

Probe name (target molecule)	**NCBI reference sequence**	**Length mRNA ol sequence**	**Probe design: number of ZZ oligoprobes**
CCL11 / Eotaxin	NM_001081871.1	514 bp	7 ZZ
CCL3 / MIP1α	NM_001114941.1	367 bp	7 ZZ
IL-4	NM_001082519.1	414 bp	9 ZZ
IL-5	NM_001082499.1	405 bp	8 ZZ
IL-13	NM_001143791.1	402 bp	8 ZZ
SCF	NM_001163962.1	1407 bp	20 ZZ
TGFβ	NM_001081849.1	1187 bp	20 ZZ
PPIB	NM_001099761.1	853 bp	19 ZZ

Pp: Base Pair.

2.9.1 RNA-ISH protocol

For the mRNA detection of the different cytokines and chemokines, an mRNA ISH manual protocol was followed (Advance Cell Diagnostics, Inc. RNAscope® 2.5 HD assay), according to the manufacturer's instructions.

Briefly, tissue sections were deparaffinised in xylene (twice, 5 minutes each), incubated in 100% ethanol (twice, 1 minute each) and air dried for 5 minutes at RT. Subsequently, sections were incubated in RNAscope® Hydrogen Peroxide (322335) (10 minutes, RT) to inhibit all endogenous peroxidase activity, washed twice in distilled water, and subjected to target retrieval pre-treatment in a pressure cooker with RNAscope® Target Retrieval Reagent (322000) (15 minutes, 100ºC). Subsequently, slides were washed twice in distilled water, incubated in 100% ethanol and air dried at RT. A hydrophobic barrier was drawn around each section with an Immedege™ pen (H-4000, Vector, Peterborough, United Kingdom). The sections were then covered with RNAscope® Protease Plus (322331), placed in a HybEZ™ Humidity Control Tray/Slide Rack (310012 and 310014), and incubated on a HybEZ™ Oven (310010 (110VAC) at 40ºC for 30 minutes to allow for cell permeabilisation and additional target retrieval. Slides were washed twice in distilled water, covered with the corresponding probes, and subjected to probe hybridisation in the HybEZ™ system (humidity control tray, slide rack and oven) at 40ºC for 2 hours. After hybridisation, slides were washed twice in RNAscope® Wash Buffer (310091) and subjected to the following incubation cycles for signal amplification in the HybEZ™ system, using the Amp reagents provided by the manufacturer for the Brown (DAB) kit (322310): Amp1 reagent (30 minutes at 40ºC), Amp2 reagent (15 minutes at 40ºC), Amp3 reagent (30 minutes at 40ºC), Amp4 reagent (15 minutes at 40ºC), Amp5 reagent (30 minutes at 40ºC), and Amp6 reagent (15 minutes at 40ºC). Between each amplification step, slides were washed twice (2 minutes each) in wash buffer. After the last wash, slides were incubated with a DAB solution for 10 minutes at RT for visualisation of the hybridised probes, and subsequently washed in tap water, and counterstained with 50% Gill's haematoxlin II (Merck, 105175). The sections were dehydrated in an ascending ethanol series (70% ethanol for 1 minutes; 95% ethanol for 1 minutes; 100% ethanol

twice, 2 minutes each), twice cleared in xylene (2 minutes each) and mounted with cover slips and xylene-based mounting medium.

A probe directed against equine PPIB (housekeeping gene coding for peptidylprolyl isomerase B) served as a positive control and was used to confirm mRNA preservation in the sections/tissue. A negative control probe directed against DapB (ACD, 310043) (bacterial gene coding for dihydrodipicolinate reductase) was also applied to all cases in order confirm the specificity of the hybridisations. A positive signal was represented by a dark brown granular reaction in the cytoplasm of the cells. A scoring system to quantify the mRNA expressed was based on the number of cells exhibiting a positive signal. It was based on the total number of positive cells/cm^2 (taking also into account the total size of the section) as score 0 (0), 0.5 (1-10), 1 (11-100), 2 (100-500), 3 (500-1000), 4 (1000-3500).

CHAPTER 3: RESULTS

3. Results

3.1 Clinical data and association with diagnosis

Overall, 153 cases met the inclusion criteria; for the majority of these horses information as to their breed, sex, age, and the location of individual lesions was available.

3.1.1 Diagnosis at the time of initial diagnostic examination

According to the diagnoses made upon the initial diagnostic examination, a total of 191 lesions were examined from the 153 horses, with a diagnosis of mast cell tumour (MCT) being made for 62 lesions and eosinophilic granuloma (EG) for 129 lesions. Of the 129 EG, 71 (72%) represented single lesions, and 48 MCT (87%) occurred as single lesions. When more lesions were observed in the same animal, they were always of the same type.

3.1.2 Breed distribution

The breed was known for 77% (118) of the cases, whereas it was not specified for 35 horses (23%). The breeds were as follows: 15 Thoroughbreds and 13 Thoroughbred cross (Thoroughbreds in total: 28/118; 27.3%); 15 Arabian and 4 Arabian cross (Arabian in total: 19/118; 16.1%). These were the most frequently represented breeds. These were followed by Warmbloods (14/118; 11.8%), 7 Welsh and 2 Welsh cross (Welsh in total: 9/118; 7.6%), Irish Sport Horse (6/118; 5%), Cob (5/118; 4.2%), Pony and Appaloosa (each 3/118; 2.5%). Two horses each were of Hanoverian, Irish Draft, Irish draft crossed with Thoroughbreds and Andalusian breed (2/118; 1.7%), and there was each one of Belgian, Holstein, Riley, CohnX, Connemara, Criolla, Dales, French Breed, Grey Pony, Icelandic, Morgan, New Forest Cross, Oldenburg, Selle Français, Shetland Pony, Show Horse, Skewbald, Spanish, Polo Pony, Sport horse, Welsh cob, Welsh cross Thoroughbreds, and Irish Sport Pony.

Among the 118 horses of which the breed was known, 75 were initially diagnosed with EG and 43 with MCT. The breeds most frequently represented among EG

cases were Thoroughbreds and Thoroughbreds crosses (16/75; 21.3%) and Warmbloods (12/75; 16%), followed by Arabian and Arabian crosses (8/75; 10.6%), Irish Sport Horse (5/75; 6.6%); Welsh and Welsh cross (4/75; 5.3%), Cob (3/75; 4%), AND Appaloosa, Pony, Hanoverian, Irish Draught (2/75; 2.6% for each). Breeds most frequently represented among MCT cases were Thoroughbreds and Thoroughbreds cross (12/43; 28%) and Arabian and Arabian cross (11/43; 25.6%), followed by Welsh and Welsh cross (5/43; 11.6%), Warmbloods and Cob (each 2/43; 4.7). The full results are tabulated in Table 6.

Table 6: Number of horses diagnosed with EEG or MCT of different breeds

Breed	EG	MCT
Thoroughbred and Thoroughbred cross	16	12
Arabian and Arabian cross	8	11
Warmbloods	12	2
Welsh and Welsh cross	4	5
Cob	3	2
Irish Sport Horse	5	1
Appaloosa	2	1
Pony	3	1
Hanoverian	2	0
Irish Draught	2	0
Irish Draught cross Thoroughbred	1	1
Andalusian	1	1
Skewbald	1	0
Belgian	1	0
Shetland Pony	1	0
Connemara	1	0
Selle Français	1	0
Oldenburg	1	0
Polo Pony	1	0
Riley	1	0
Morgan	1	0
Criolla	1	0
New Forest cross	1	0
Sport horse	1	0
Dales	1	0
Icelandic	1	0
Spanish	1	0
Show Horse	0	1
Holstein	0	1
French Breed	0	1
Irish Sports Pony	0	1
Cohn-X	0	1
Welsh cross Thoroughbred	0	1
Welsh cob	1	0

3.1.3 Age distribution

Animals included into the study ranged in age from 2 to 25 years, with a mean age of 3 years. For animals with a diagnosis of EG the age ranged from 2 to 23 years, with a mean age of 9 years. For animals with a diagnosis of MCT, ages ranged from 3 to 25 years, with a mean age of 11.3 years.

For Thoroughbred and Thoroughbred cross with a diagnosis of EG, the recorded ages ranged between 2 and 20 years (mean age: 9 years) and between 9 and 21 years (mean age: 13 years). For Arabian and Arabian cross with a diagnosis of MCT, the ages ranged from 5 to 23 years (mean age: 13.5 years) with a diagnosis of EG, between 3 and 21 years (mean age: 10 years) .For Warmblood, the age ranged between 5 and 18 years (mean age: 7.3 years) with a diagnosis of EG, and between 4 and 18 years (mean age: 11 years) for MCT; Welsh and Walsh cross, the age ranged between 8 and 22 years (mean age: 9.5 years) with a diagnosis of EG, and between 7 and 13 years (mean age: 9.5 years) for MCT.

In addition, for breeds affected with EG only, the age range 6-10 years for Cobs (mean age: 8 years), 10 -20.5 years for ponies (mean age: 15.2 years), 9.6-13 years for Irish (mean age: 7.5 years), 7-11 years for Appaloosas (mean age: 9 years), 6-7 years for Hanoverians (mean age: 6.5 years), and 5-9 years for Irish Draughts (mean age: 7 years).

The age of each one horse affected by MCT (Cob, Pony, Irish, Appaloosa, and Andalusian) was 12, 11, 13, 10 and 6 years, respectively. For Hanoverian and Irish Draught, no MCT was diagnosed, and the age was not available for the Andalusian. For Irish draft crossed with Thoroughbreds and Andalusian, one horse was affected with EEG and ages were 7 and 6 years, respectively.

The data also for other breeds (one horse for each) were grouped together and showed the following age: age range between 4 and 15 years (mean age: 8.3 years) for EG (Skewbald, Belgian, Grey Pony, Shetland Pony, Connemara, Selle Français, Oldenburg, Polo Pony, Riley, Morgan, Criolla, New Forest, Cross Sport horse, Dales, Icelandic, Spanish, Welsh cob); ages ranged between 7 and 22 years (mean age: 14 years) for MCT (Show Horse, Holstein, French Breed, Irish Sports Pony, Cohn-X, Welsh cross Thoroughbred). A summary is provided in Table 7.

Table 7: Age (mean and range) in each breed with both types of lesions

Breed	EG age (years)		MCT age (years)	
	Mean	**Range**	**Mean**	**Range**
Thoroughbred/cross	9	2-20	13	9-21
Arab/cross	13.5	5-23	10	3-21
Warmblood	7.3	5-18	11	4-18
Welsh/cross	13	7-22	9	7-13
Cob	8	6-10	* 12	
Pony	15.2	10-20.5	* 11	
Irish	7.5	9.6-13	* 13	
Appaloosa	9	7-11	* 10	
Hanoverian	6.5	6-7	“	
Irish Draught	7	5-9	“	
Irish Draught cross Thoroughbred	* 7		* 6	
Andalusian	* 6		“	“

Both types of lesions (EG and MCT) after initial diagnosis; * = one case recorded, “= no case recorded

3.1.4 Sex distribution

The sex was known for 142 horses out of the total of 153. Of those where the sex was known, 90/142 (63%) were male and 52/142 (37%) were female. Horses diagnosed with MCT were mainly males (65%; 36/55), the difference between sexes was less evident for horses with EG, where 55% (54/98) were male (Table 8).

Table 8: Summary of the case material selected on the basis of the initial diagnosis

	Total number (%)	MCT	EG
Horses	153	55	98
Male	90 (63%)	36 (65%)	54 (55%)
Female	52 (37%)	14 (25%)	38 (39%)
Not stated	11 (7%)	5 (10%)	6 (6%)

Comparing single and multiple lesions, the sex distribution was similar: the number of males (39/67; 58.2%) was similar to females (28/67; 41.8%) in single lesions; this was comparable with the results obtained for those horses that exhibited multiple lesions (males: 15/25; 60%; 10/25; 40%).

In MCT there were 44 single lesions; males (33 /44; 75%) were overrepresented in exhibiting single lesions, whereas multiple lesions seemed to be equally distributed between the sexes (males: 4/7, 57%; females 3/7, 43%).

3.1.5 Location of lesions

The lesions diagnosed as EG were distributed among the body as follows: trunk (18/98; 18.4%), head (6/98; 6%), eyelid and limbs (3/98; 3% for each location); conjunctiva (1/98; 1%). Five horses had more than one lesion. These were distributed as follows: eyelid and conjunctiva; neck, trunk and head; neck and trunk; eye, neck and trunk; eyelid and shoulder. Information on the location was not available for 63.2% (62/98). MCT were widely distributed across the body but most commonly reported on the head (14/55; 25.4%), and eye (10/55; 18.2%, 5 each at conjunctiva and eyelid), trunk (5/55; 9%), neck (3/55; 5.5%) and limb (3/55; 5.5%). In 20 horses (36.4%), information on the location of the legion[s] was not available.

3.1.6 Size of lesions

The major diameter of EG clinically investigated at the time of surgical procedure/sampling and reported with clinical information ranged from 5mm to 9cm, with a mean major diameter of 2.4cm. For MCT, the size ranged from 2mm to 10cm, with a mean major diameter of 2.3cm. For 19/98 EG and 8/55 MCT, information on size was not available.

3.2 Histopathological examination and case review

Based on the hypothesis that, to date, there has been no definite proof that EG and MCT in the skin of horses are two different entities, and in order to establish clear diagnostic guidelines, all cases were re-examined, with the examiner being 'blinded' in that she did not have access to the initial diagnoses.

The morphological examination revealed variable histological features (Table 9).

Assessment of the dermal location showed that the majority of lesions involved the mid 98/191 (51%) and/or the deep 123/191(64%) dermis, whereas only 47/191 (25%) involved the superficial dermis and 41/191(22%) the subcutis. As many as 93/191 (49%) lesions occupied more than one layer.

Of the 191 lesions, the great majority 176/191 (92%) exhibited areas consistent with EG; 15/191 (8%) did not exhibit any evidence of EG components (EGC). By comparison, only 76/191(40%) showed mast cell aggregates (MCA), suggesting that 115 /191 (60%) cases were EG only. In cases with MCA, the size and number of EG areas and MCA varied gradually within the population without a clear cut off.

Individual mast cells were found distributed throughout the lesion in 32/191 (17%) of all lesions. In 55/176 (31%) of the cases with an EGC there was evidence of EGC association with hair follicles, and in 122/176 (69%), the EGC seemed to contain embedded collagen. Mineralisation was observed in 67/191 (35%) of all lesions. A few cases 31/191 (16%) exhibited ulceration of the epidermis. All lesions with MCA exhibited infiltrating eosinophils within the MCA; this was variable and mild (1%-20% of the area represented by infiltrating eosinophils on average) in 35 lesions, moderate (20%-50% of the area represented by infiltrating eosinophils on average) in 22, and marked (50%-95% of the area represented by infiltrating eosinophils on average) in 19 lesions. Lesions with MCA were assessed for proliferation of the mast cell population; in 64/76 (84.2%), mitotic figures were observed. 70/191 (37%) of the lesions exhibited lymphocyte aggregates associated with the lesions.

Notably, there were four lesions that exhibited unique morphological features characterised by marked eosinophil infiltration (95% of the area represented by infiltrating eosinophils on average) and an embedded mast cell population that was characterised by large pleomorphic mast cells. These are described separately from the study population and are referred to as histiocytic-like atypical mast cell tumours (see APPENDIX II - section B). The results of the morphological evaluation of the 191 cases are summarised in Table 9.

Table 9: Summary of morphological finding, based on HE and TB stain for 191 lesions

Histological Features	**Number**	**Percentage**
Location		
Superficial dermis	47/191	25%
Mid dermis	98/191	51%
Deep dermis	123/191	64%
Subcutis	41/191	22%
Presence of EG/eosinophils and MCT/MCA		
EG	176/191	92%
MCT and/or mast cell aggregates (MCA)	76/191	40%
Pure MCT (without EG component)	15/191	8%
No evidence of MCA	115/191	60%
Scattered individual mast cells	32/191	17%
EGC associated with hair follicles	55/176	31%
Infiltrating eosinophils in MCA	76/76	100%
Mineralisation	67/191	35%
Ulceration	31/191	16%
Collagen in EG areas	122/176	69%
Proliferation of mast cells (mitotic figures) in cases with MCA	64/76	84%
Lymphocyte aggregates	70/191	37%
Cases with dominance of mast cells with macrophage/histiocyte appearance (atypical cases)	4/191	2%

3.2.1 Selection of the case material for detailed investigations and development of a system to classify the lesions based on the histological features

From all of the 191 examined cases, 95 lesions were selected based on their tissue preservation, the completeness of the lesions on the section, and the presence of surrounding normal tissues. These 95 cases were selected for further analysis (Appendix II - section A).

The presence and relationship between the EGC and MCA components was used to establish five morphological categories, to which all lesions were subsequently allocated regardless of initial diagnosis. This also conferred a (new) diagnosis. The five categories and their basic criteria were as follows:

- Category I: Areas of EGC, without MCA
- Category II: EGC and MCA present; EGC total area larger than MCA area
- Category III: EGC and MCA present; EGC total area equal to MCA area
- Category IV: EGC and MCA present; EGC total area smaller than MCA area
- Category V: MCA only

Based on these categories, three major groups (and diagnoses) were created:

- Group 1: Cases with EGC only (Category I)
- Group 2: Cases with EGC and MCA (Categories II+III+IV)
- Group 3: Cases with MCA only (Category V)

Based on these three groups, the following two diagnoses were established:

- Eosinophilic granuloma (EG): Group 1 (i.e. Category I)
- Mast cell tumour (MCT): Groups 2 and 3 (i.e. Categories II-V)

After the establishment of the five categories, the 95 cases selected for further investigations were allocated to the different categories; 31 to Category I, 14 to Category II, 13 to Category III, 23 to Category IV, and 14 to Category V.

Among these cases were 11/95 (~10%) in which the (new) diagnosis did not match with the initial diagnosis. Ten cases previously diagnosed as EG were reclassified following the new classification as MCT based on the identification of MCA within large EG areas (Groups 2 and 3). Of these, seven were assigned to Category II, and one each to Categories III, IV and V. One single lesion that was previously diagnosed as MCT was reclassified as EG and fell into Category I based on (TB) stain that lesion lack evidence of MCA. Information on all individual cases is presented in Appendix II – section A.

3.3 Clinical data for groups and diagnoses

The 95 lesions were from 78 horses. The breed was known for 62 of these animals. There were 43 males and 30 females (for five horses, information on the sex was not available), and the age ranged from 3 to 25 years (mean age: 11 years). For all further assessments, the lesions were arranged in Groups (1-3) and according to the final diagnosis (EG and MCT).

3.3.1 Breed distribution

In Group 1 (EG), the breed was given in 23 of the 30 horses. Most frequently represented were Thoroughbreds and Thoroughbreds crosses (8/23; 34.7%), Arabian and Arabian crosses (3/23; 13 %), followed by Warmbloods (2/23; 8.7%) and Cobs (2/23; 8.7%). There was one each of; Irish Draught, New Forest Cross, Oldenburg, Selle Français, Spanish, Irish Sport, Welsh cob, and Irish draft crossed with Thoroughbred.

In Group 2 (EGC with MCA), the breed was given in 34/42 horses. Most frequently represented were Arabian and Arabian crosses (10/34; 29.4 %) and Thoroughbreds and Thoroughbreds crosses (5/34; 14.7 %), Welsh with Welsh cross (5/34; 14.7%) followed by Appaloosa (2/34; 6 %), Pony (2/34; 2%) and Warmbloods (2/34; 6%). There was one each of; Show horse, Welsh crossed with Thoroughbred, Irish draft crossed with Thoroughbred, Holstein, Andalusian, French breed, Cob and Cohn-x.

For Group 3 (pure MCT) the breed was known for 5 of the 6 horses. Most of the 5 were Arabian with Arabian crosses (3/5; 60%), with the other two horses being a Thoroughbred cross, and an Appaloosa.

Looking at the diagnosis of MCT (Groups 2 and 3), the breed was known for 39 of the 48 horses. The breed distribution among the newly categorised EG (Group 1) and MCT (Groups 2 and 3) is summarised in Table 10.

Table 10: Breed distribution among EG (Group 1) and MCT (Groups 2 and 3)

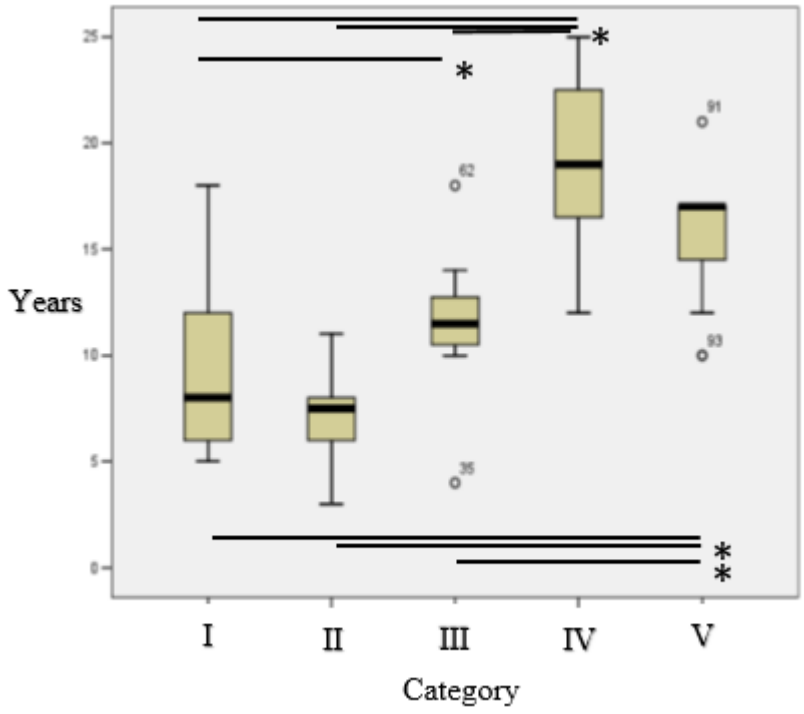
Breed	EG	MCT
Andalusian	0	1
Cob	1	0
Appaloosa	0	3
Arab	2	13
Arab cross	1	3
Cob	1	0
Cob Cross	0	1
Cohn-X	0	1
French Breed	0	1
Grey Pony	0	1
Holstein	0	1
Irish draft cross	1	1
Irish Draught	1	0
Irish Sports	1	0
New Forest cross	1	0
Oldenburg	1	0
Pony	0	1
Selle Français	1	0
Show Horse	0	2
Spanish	1	0
Thoroughbred	4	2
Thoroughbred cross	4	12
Warmblood	3	2
Welsh	0	1
Welsh cross	0	5
Welsh Cob	1	0
Welsh cross Thoroughbred	0	2
Unknown	7	9
Total	31	64

Arabs with Arab cross as well as Thoroughbred with thoroughbred cross were overrepresented in MCT compared to EG. There was no statistical association between Thoroughbred and MCT while a significant increase in the number of Arabian exhibiting MCT was found.

3.3.2 Age distribution

For Group 1, the age of affected horses ranged from 5 to 18 years, with a mean of 9 years. For Group 2, the age ranged from 3 to 25 years (average: 12 years), whereas in Group 3, affected animals ranged between 12 and 21 years of age, with a mean age of 15 years. Considering the separate categories there was a significant increase in the mean age of animals within Groups IV and V with lesions compared to Groups I and II (Figure 4).

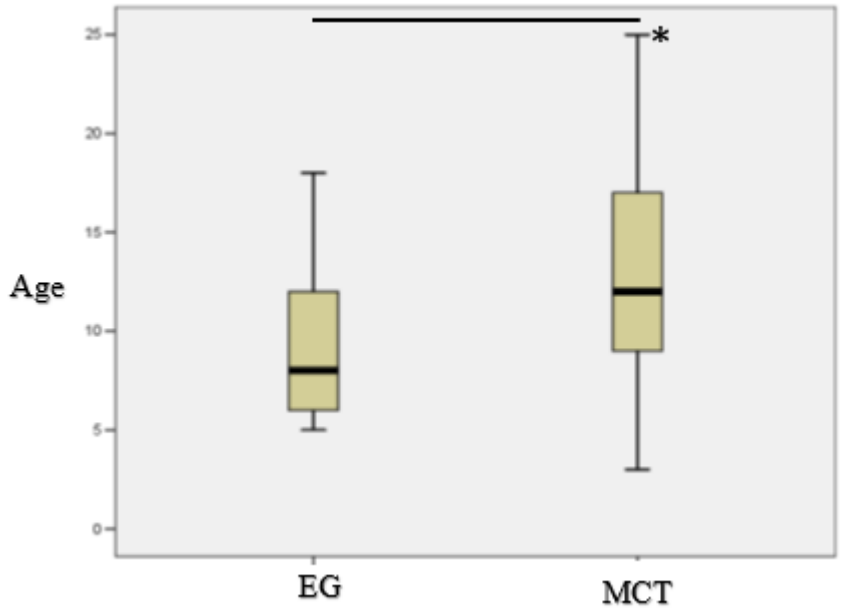
Figure 4: Box and whisker plots showing the difference in age among categories



*=P<0.05, **=P<0.01**,** Statistical test (Mann-Whitney U)

In addition, horses with EG (Group 1) were significantly younger than horses with MCT (Groups 2 and 3) as shown in Figure 5.

Figure 5: Box and whisker plots show the difference in age between EG and MCT



*=P<0.05, Statistical test (Mann-Whitney U)

3.3.3 Sex distribution

For group 1 lesions, the sex was known for 28 out of the 30 affected horses; there were 14 affected male horses and 14 affected female horses. Among the 41/42 horses with group 2 lesions with known sex, the majority (23; 55%) were male, with 18 being female (45%). All six animals with group 3 lesions were males. With reference to MCA there were 29 male horses and 18 female horses.

Horses with MCT (groups 2 and 3) were, in the majority, male (42/64; 65%) with only 19 females and three with unknown sex. Despite the 3 to 1 ratio of males with a diagnosis of MCT, the difference was not found to be significant.

3.3.4 Location of the lesion

For Group 1 lesions (EG), information on the location was only available for 9/31 lesions (29%). Single lesions originated from the trunk (6/31; 19.3%) and

conjunctiva (1/31; 3.2%). In two horses with more than one lesion they were reported to originate from the eyelid and conjunctiva as well as the neck and trunk.

For Group 2 lesions, information on the location was available for 27/50 lesions (54%). They were located on the head (17/50; 34%), trunk (3/50; 6%), leg 2/50, neck (2/50; 4%), eyelid (2/50; 4%), and conjunctiva (1/50; 2%).

For Group 3 lesions, information on the location was available for 4/14 lesions (29%). They were located on the head (2/14; 14.3%) and eyelid (2/14; 14.3%).

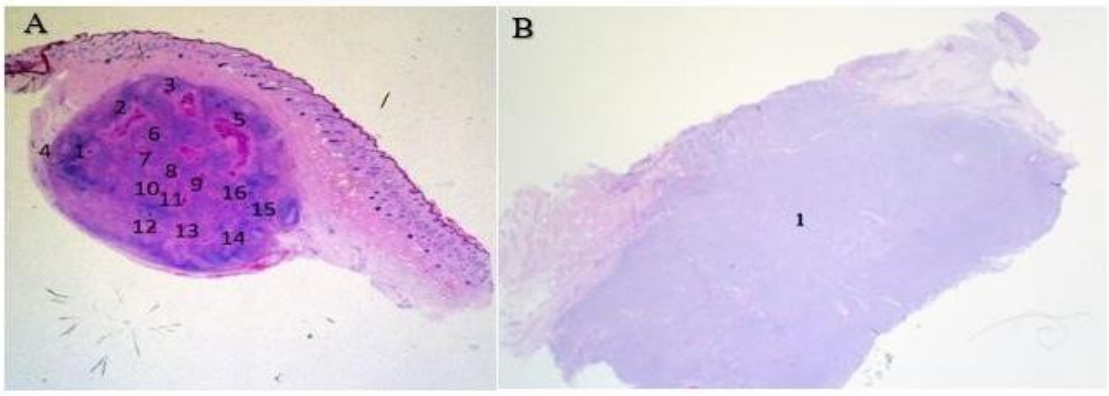
For MCT (Groups 2 and 3), lesions were located on the head (19/64; 30 %), trunk (3/64; 5%), leg 2/64, neck (2/64; 3%), eyelid (4/64; 6%), and conjunctiva (1/64; 2%).

3.4 Assessment of morphological features in Categories (I-V)

3.4.1 Size (area) of the lesions and proportion (area) of EGC and MCA within lesions

The size and number of EGC and MCA were evaluated by counting the number of EGC present in the lesion and measuring the area (pixels) occupied by each EGC and MCA. This was achieved using low power scanning at the same magnification and measured using computer software (Figure 6).

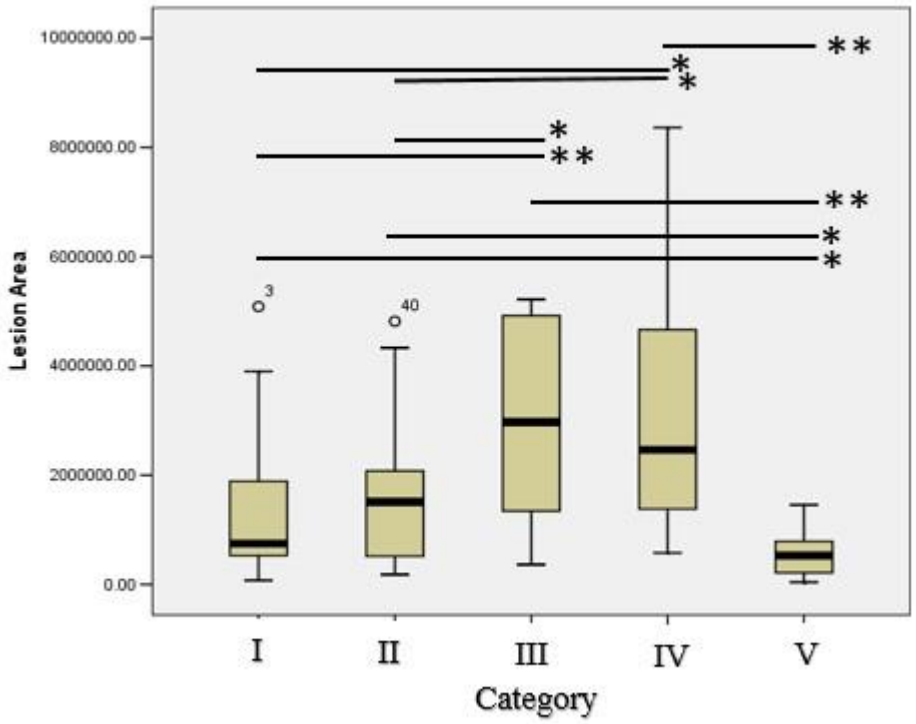
Figure 6: Representative picture of low power scanning of two sections



Labels indicate the objects where the area for EGC (A) Case No. 09L-3297 EG (Category I) and MCA (B) Case No. 07L-3494 MCT (Category V) was assessed.

Considering all the lesions, the pure MCT (Category V) were significantly the smallest lesions, while MCT in Category III and IV were the largest (Figure 7).

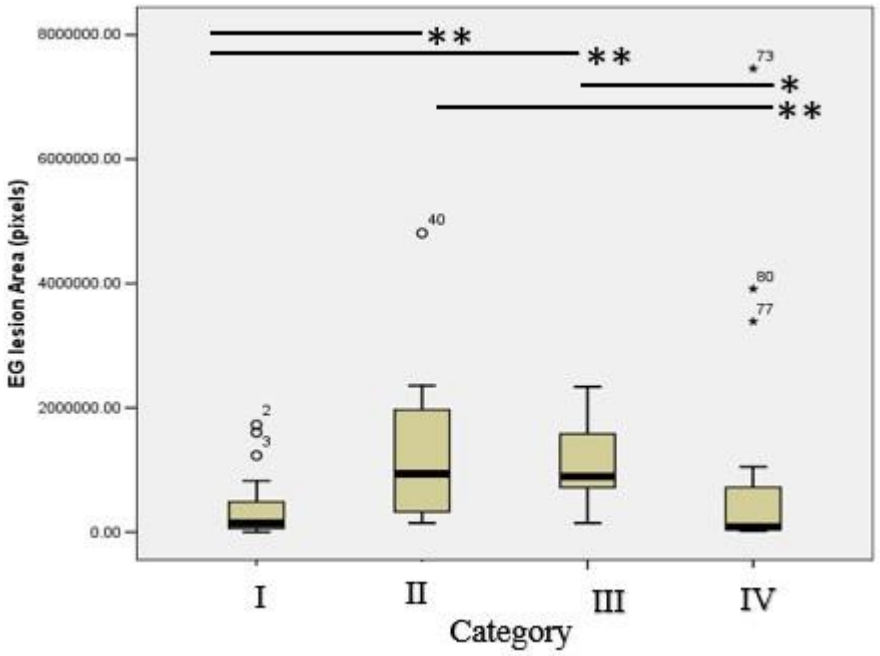
Figure 7: Box and whisker plots showing the difference in area (pixels) of lesions of the different categories



*=P<0.05, **=P<0.01. Statistical test (Mann-Whitney U) and the numbers representing outliers.

Considering the area of EGC, their size was larger in Categories II and III compared to IV, as expected (Figure 8).

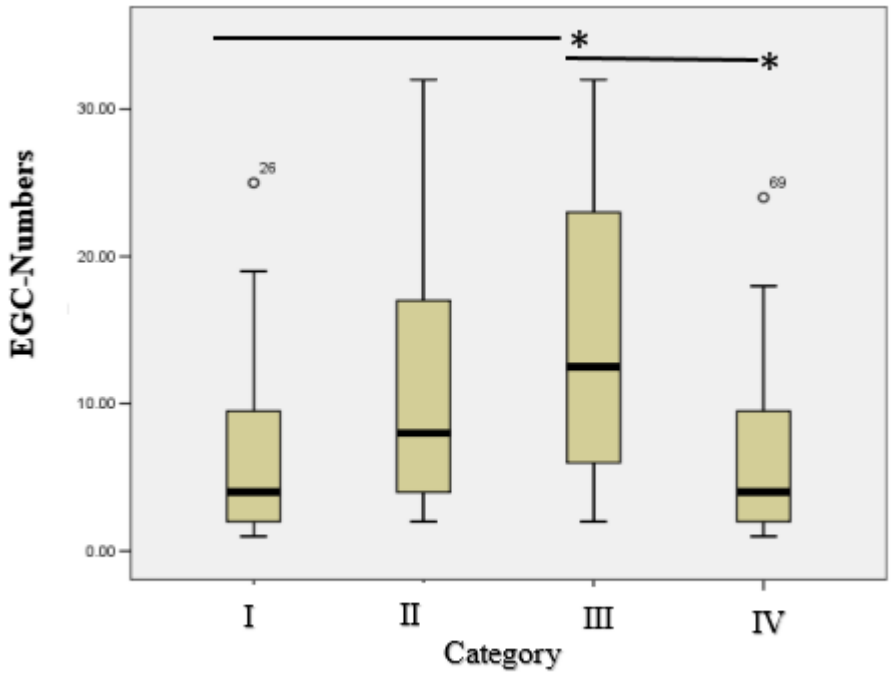
Figure 8: Box and whisker plots showing the difference in EGC area (pixels) in lesions of the different categories



*=P<0.05, **=P<0.01. Statistical test (Mann-Whitney U) and the numbers representing outliers.

The number of EGC within a lesion was variable within the categories but only a weaker statistical association was found in Category III which contained more EGC areas than lesions of the other categories (Figure 9).

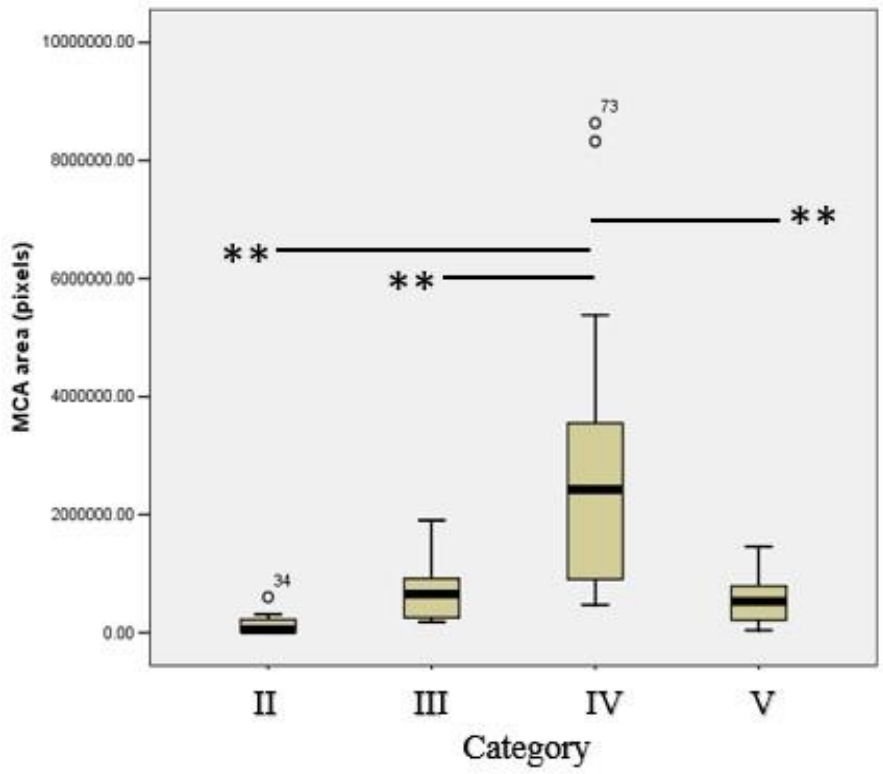
Figure 9: Box and whisker plots showing the difference in EGC numbers between the different categories



*=P<0.05. Statistical test (Mann-Whitney U) and the numbers representing outliers.

When the MCA area was measured, there was a significant increase in MCA size in Category IV with a possible trend from Categories II to IV. MCA in Category V were significantly smaller in size (Figure 10).

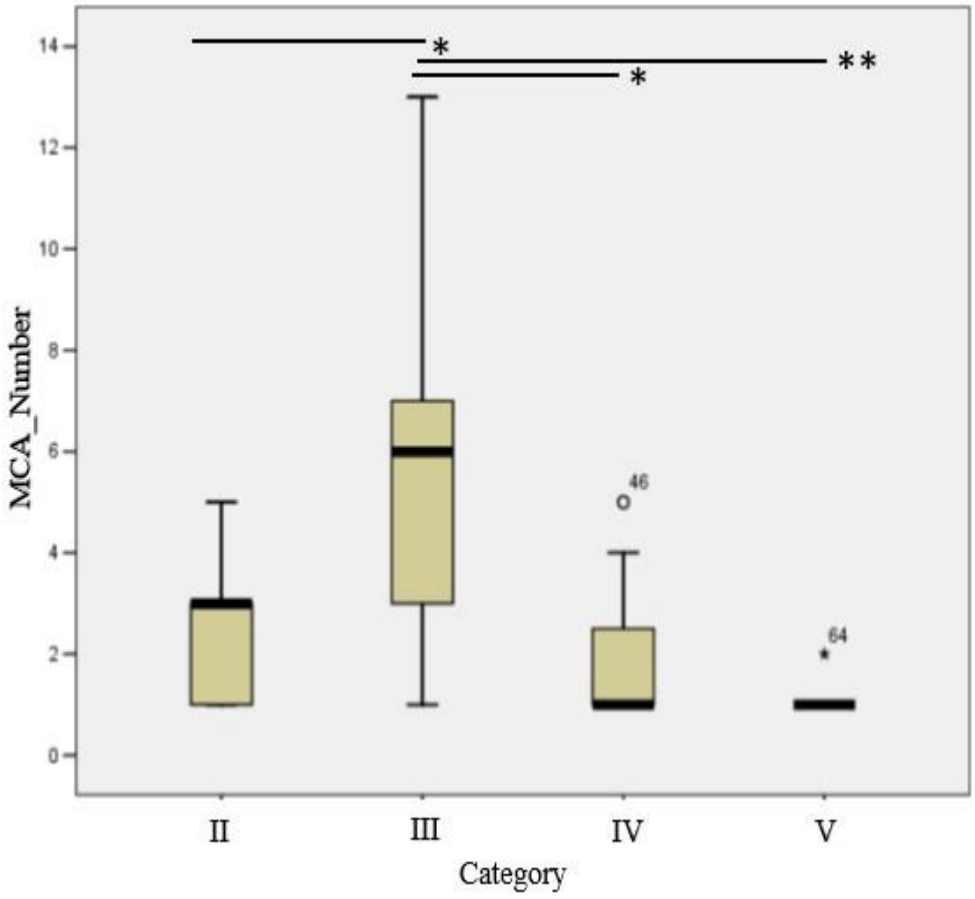
Figure 10: Box and whisker plots showing the difference in MCA area (pixels) between the different categories with MCA



**=P<0.01. Statistical test (Mann-Whitney U) and the numbers representing outliers.

The number of MCA among groups was similar between Categories II and IV, while there was a significant increase in the number in Category III. In contrast, pure MCT (Category V) were almost exclusively comprised of one single MCA (Figure 11).

Figure 11: Box and whisker plots showing the difference in MCA numbers between the different categories



*=P<0.05, **=P<0.01. Statistical test (Mann-Whitney U) and the numbers representing outliers.

3.4.2 Location of lesions within skin and subcutis

Lesions often involved more than one location (65/95; 68.4%). A comparison of the five categories with regards to the location (i.e. involvement) of the lesions in the skin and subcutis showed that EG (Group 1; Category I) were more frequently located in the superficial dermis than lesions containing MCA (Categories I-IV). Interestingly, pure MCT (Group 3; Category V) were exclusively located in the dermis, mainly the mid and deep dermis. Involvement of the subcutis increased from Categories I to IV. Overall, the deep dermis was involved most commonly (Figure 12 and Table 11).

Figure 12: Histogram showing the percentage of lesions involving a specific layer for each category (a single lesion can extend to more than one layer)

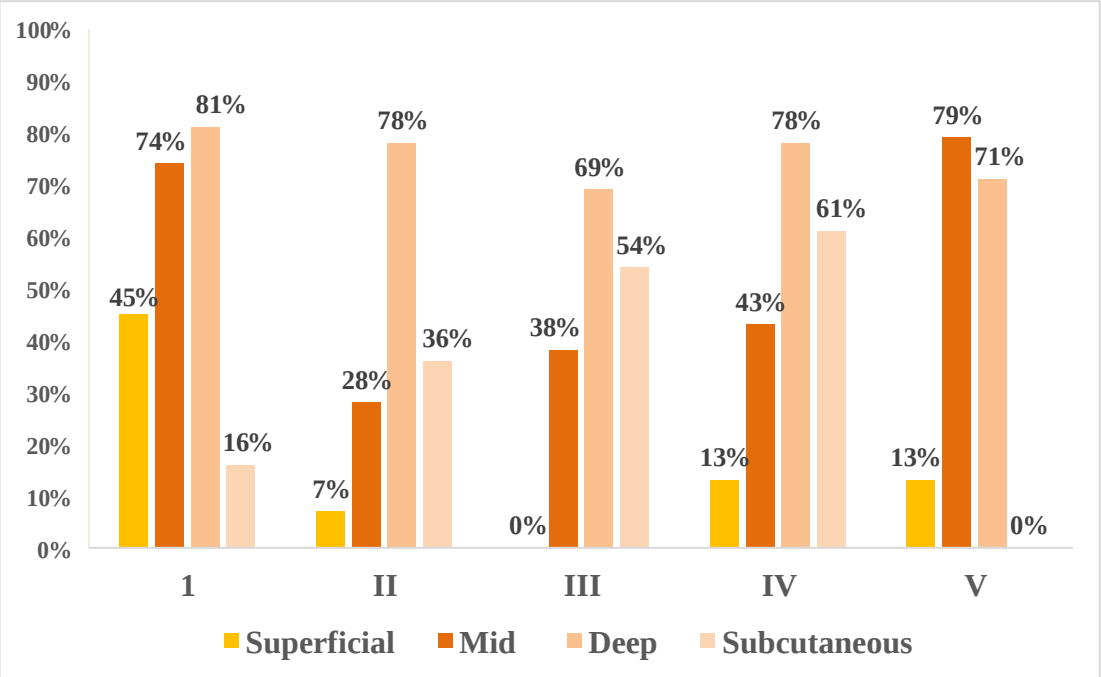
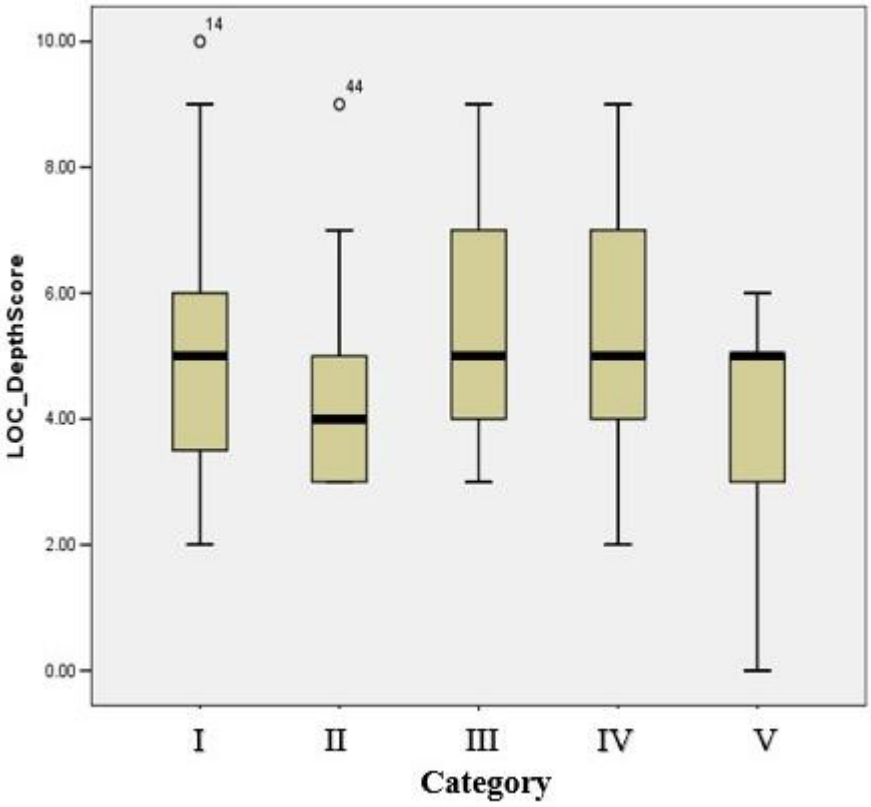


Table 11: Location of lesions in the different categories in the dermis and subcutis

Location	**No. of total**	**Category**				
		I (n=31)	**II** (n=14)	**III** (n=13)	**IV** (n=23)	**V** (n=14)
Superficial dermis	21/95	45% (14)	7% (1)	0	13% (3)	21% (3)
Mid dermis	53/95	74% (23)	28% (4)	38% (5)	43% (10)	79% (11)
Deep dermis	73/95	81% (25)	78% (11)	69% (9)	78% (18)	71% (10)
Subcutis	31/95	16% (5)	36% (5)	54% (7)	61% (14)	0

Within all the lesions examined, 65 involved more than one layer; Category I (24/31: 77.4%), Category II (7/14; 50%), Category III (7/13; 54%), Category IV (17/23; 74%), Category V (10/14; 71.4%). When the "depth score" was applied to the lesions, which weighed the involvement of multiple layers, no significant differences were found between the five categories (Figure 13).

Figure 13: Box and whisker plots to compare the depth scores between lesions of the different categories

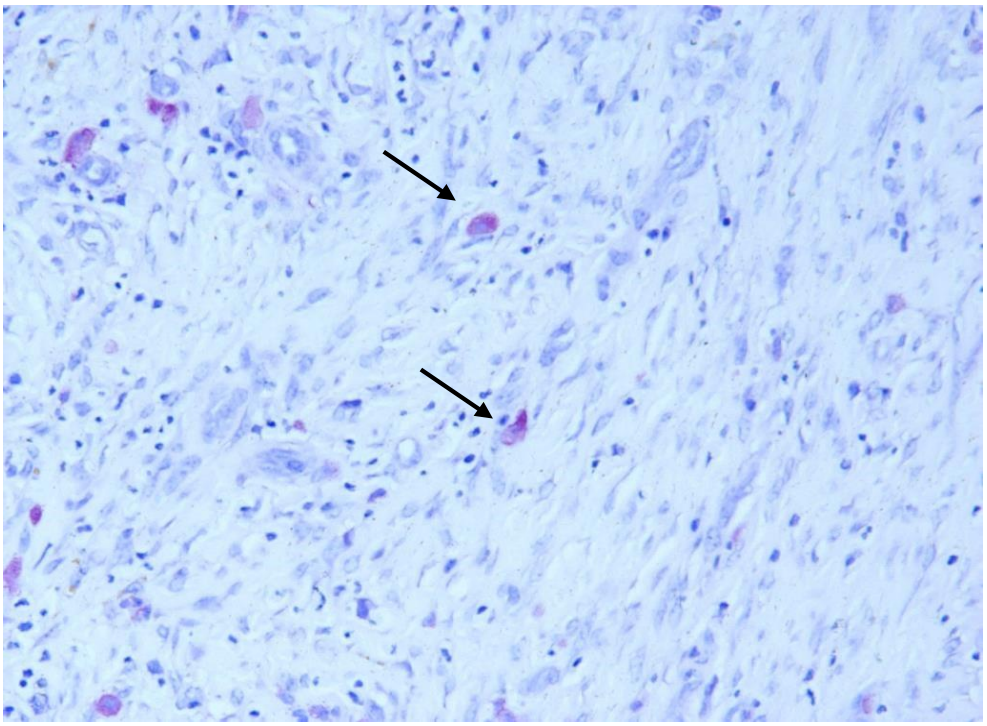


*=P<0.05, **=P<0.01. Statistical test (Mann-Whitney U) and the numbers representing outliers.

3.4.3 Presence of individual mast cells disseminated in the lesions

Individual mature mast cells (not forming aggregates) were found disseminated throughout the lesions in all 14 cases (100%) of Category II. They were also observed in 42% (13/31) of the pure EG (category I), but were absent in the lesions noted within Categories III to V (Figure 14).

Figure 14: Case No. 11L-2439 EG (Group 1, Category I): Individual mast cells (arrows) are found scattered within the lesion. Toluidine blue stain, 400x.

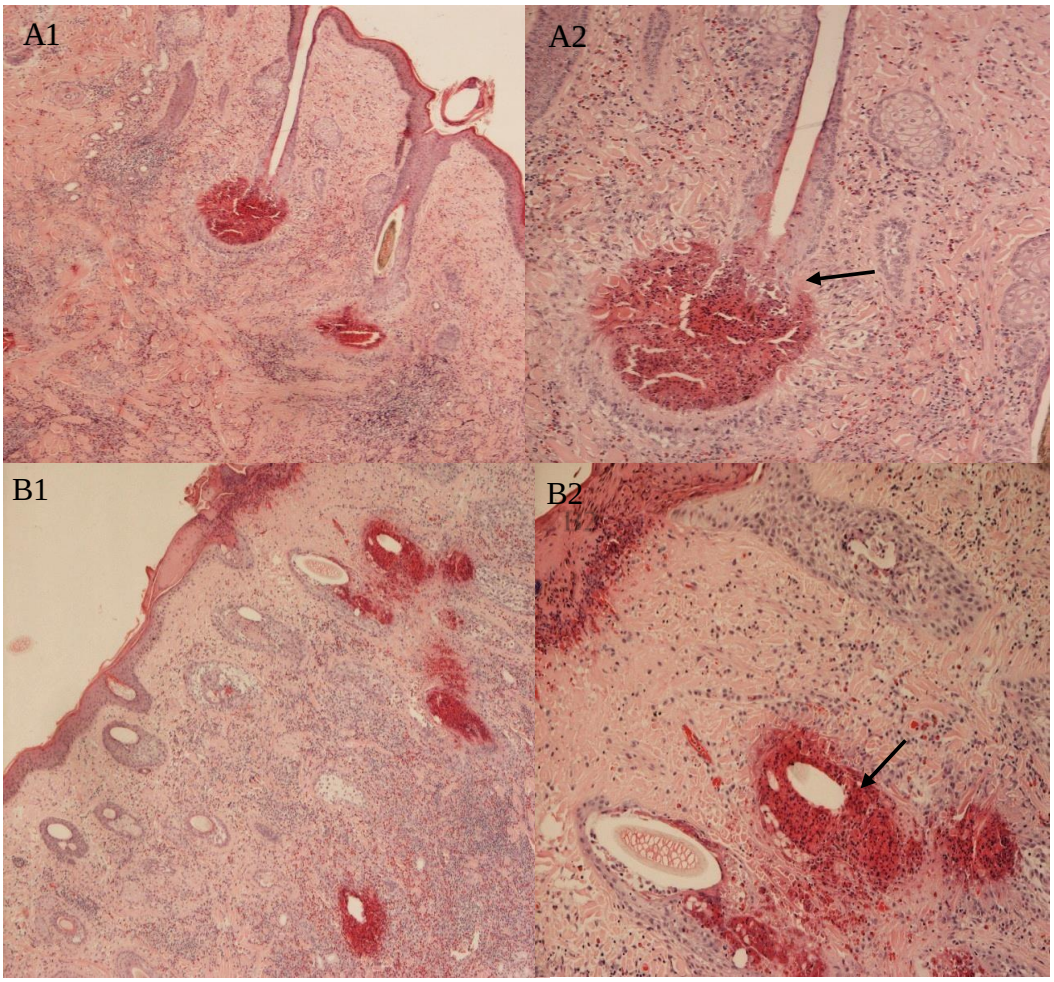


3.4.4 EGC in association with hair follicle structures

In almost half of the EG (16/31; 52%; Category I, Group 1) the EGC involved hair follicles, often effacing the hair follicle in a "furunculosis-like" pattern (Figure 15).

The statistical analysis confirmed a highly significant (P<0.001) association of EGC in pure EG (Group 1) with hair follicles compared to EGC in MCT (Categories II-IV; Groups 2 and 3).

Figure 15: Case Nos. 14L-4109 (A) and 11L-0931 (B)

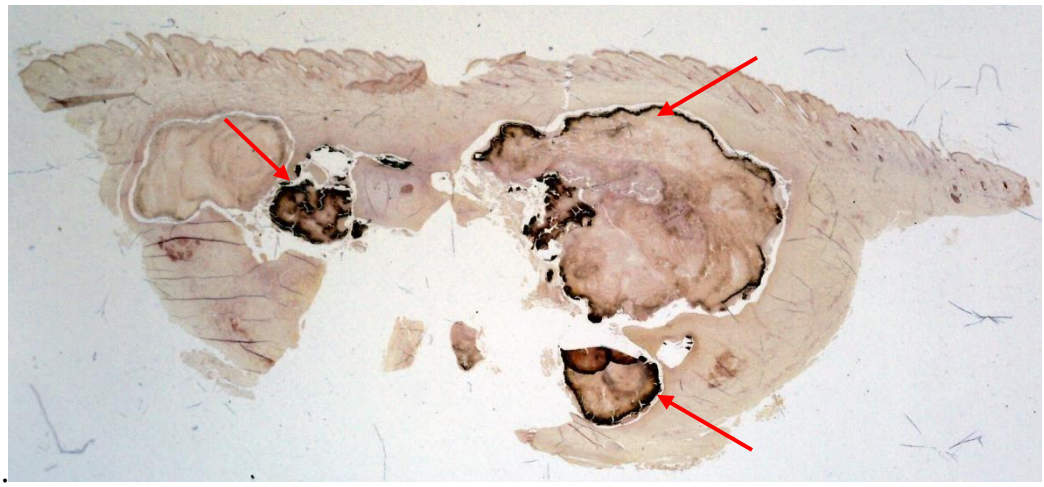


Association between EGC and hair follicle in two cases. Low power (A1 and B1) and high power (B2 and B2) views of EGC involving hair follicles (arrows). HE stain, 100x (A1, B1) and 400x (A2, B2).

3.4.5 Mineralisation

Focal areas of mineralisation were identified within EGC. Assessment of HE-stained sections identified focal areas of mineralisation in 64.5% of lesions in Category I, 8% of lesions in Category III and 21% of lesions in both Categories II and IV. Mineralisation was not identified in the pure MCT (Category V) (Table 12), as these did not contain areas of EGC. Using the von Kossa stain to specifically illustrate calcium salts (Figure 16), it became obvious that even more lesions exhibited focal areas of mineralisation (a total of 64 lesions). In Categories I to IV, their frequency varied between 61% and 100%. However, Category V lesions were confirmed to be free of mineralisation.

Figure 16: Case No. 05L-1008. Case of MCT with EGC (Category II)



The low power scan illustrates the presence of several focal areas of mineralisation (arrows). Von Kossa stain, 10x.

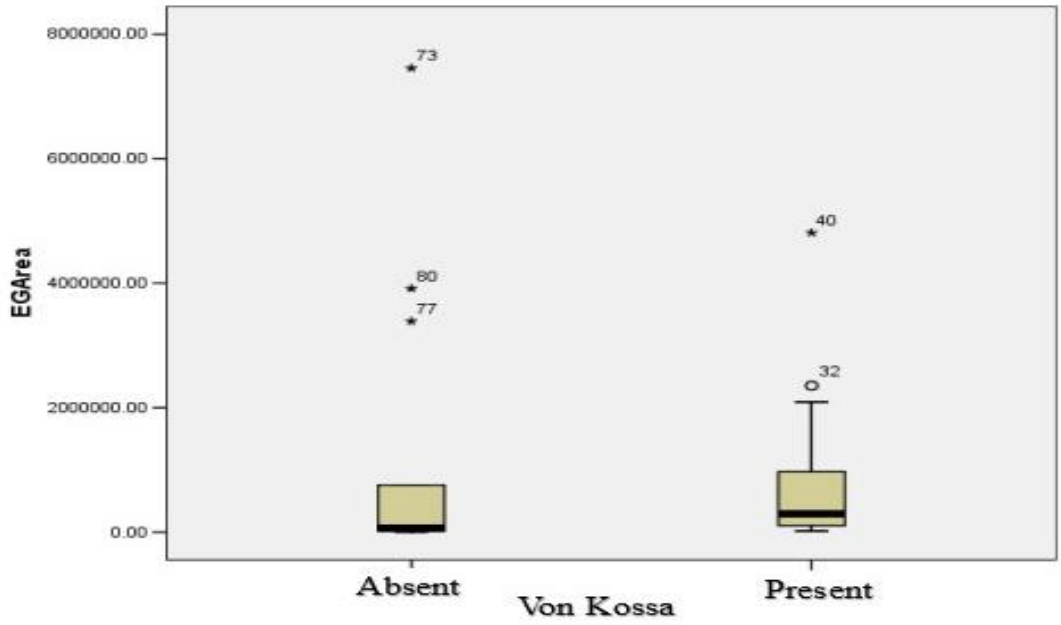
Table 12: Frequency of focal mineralisation in EG (Category I) and MCT comprised of MCA and EGC (Category II-IV), as identified in HE- and von Kossa-stained sections

Mineralisation, identified in	**No. of total**	**Category**			
		I (n=31)	**II** (n=14)	**III** (n=13)	**IV** (n=23)
HE stain	27/81	64.5% (20)	21% (3)	8% (1)	21% (3)
Von Kossa stain	64/81	84% (26)	100% (14)	77% (10)	61% (14)

Based on the results obtained from the von Kossa-stained sections, there was a significant association between the frequency of mineralisation in Group 1 (EG) lesions compared to the other groups ($P<0.001$); this was lacking in MCT (Groups 2 and 3).

When the presence of mineralisation was correlated with the average size of the EGC lesion, no statistical association was found (Figure 17). This suggests that mineralisation is not dependent on the size of an EGC area.

Figure 17: Box and whisker plots showing the lack of association between EGC area (pixels) and presence or absence of mineralisation



Statistical test (Mann-Whitney U) and the numbers representing outliers.

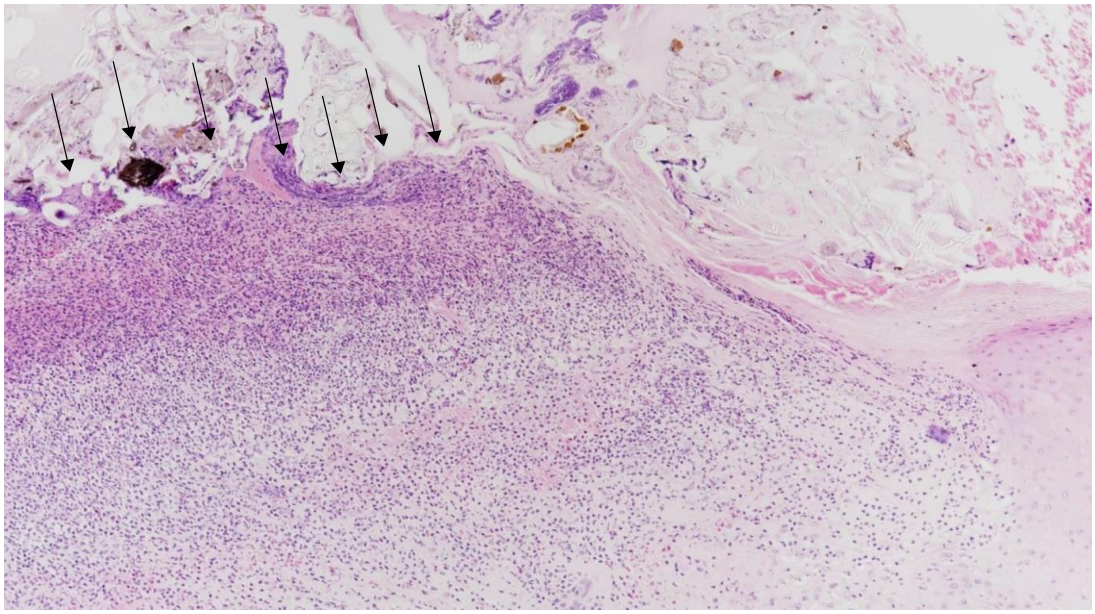
3.4.6 Ulceration

As shown in Table 13 and Figure 18, in a few cases within each category, ulceration was seen without statistically significant differences between the groups. Ulceration was not always directly associated with the lesions, suggesting it was mechanically induced.

Table 13: Presence of ulceration in lesions of all five categories

Ulceration, identified in	**No. of total**	**Category**				
		I (n=31)	**II** (n=14)	**III** (n=13)	**IV** (n=23)	**V** (n=14)
HE stain	13/95	13% (4)	14% (2)	15% (2)	13% (3)	14% (2)

Figure 18: Case No. 06L0445, Category V



Focal area of ulceration in one representative lesion (arrows). HE, 200x.

3.4.7 Presence of collagen in EGC areas

Among all types of lesions that contained EGC (Categories I to IV) were some cases that exhibited evidence of collagen fibres within areas of EGC in the HE stained sections. This was further assessed in the Masson Trichrome-stained sections which confirmed the presence of collagen fibres (or fragments) in these areas in a large proportion of lesions (Figure 19). They were very frequent in lesions of Categories I and II, but rather rarer in the other Categories (III and IV) (Table 14). The

difference in the results obtained from HE stain and Masson's Trichrome stain was due to the different methodological approaches that they use to identify collagen fibres/fragments. The HE stain relies on the morphological identification of fibrillar eosinophilic material (which can be misinterpreted as other material such as fibrin). In contrast, the Masson's Trichrome stain identifies collagen more precisely as green stained fragments, but since some collagen fibres may stain red (in particular when degenerated) this could lead to possible underestimation.

Figure 19: Case No. 09L-3757, Category II. Fragments of collagen fibres within an EGC area (arrows). Masson Trichrome stain, 400x

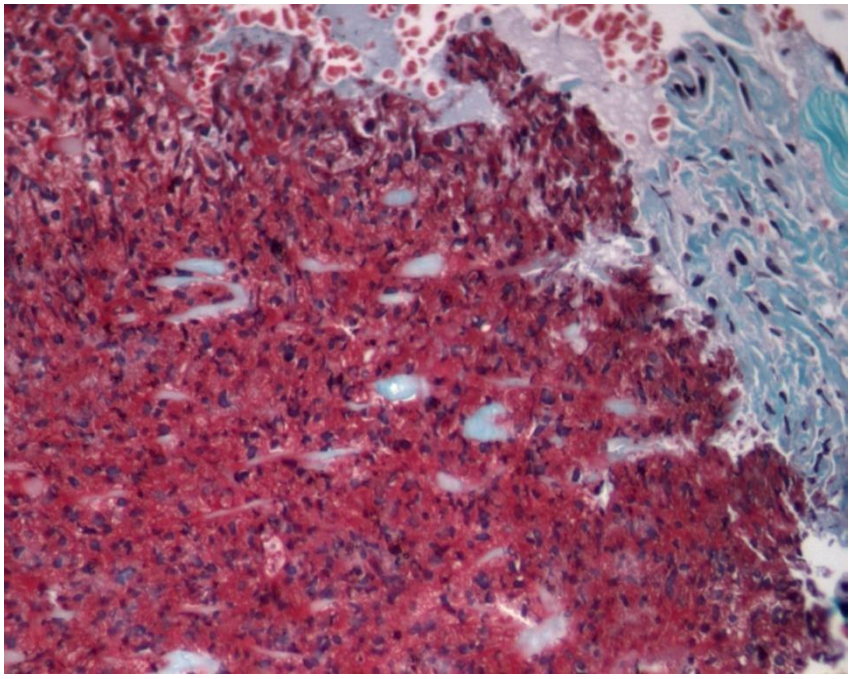


Table 14: Presence of collagen within EGC areas in the five categories, based upon HE and Masson trichrome stain

Collagen in EGC, identified in	**No. of total**	**Category**			
		I (n=31)	**II** (n=14)	**III** (n=13)	**IV** (n=23)
HE stain	56/81	81% (25)	100% (14)	85% (11)	26% (6)
Masson Trichrome stain	57/81	93% (29)	93% (13)	46% (6)	40% (9)

The statistical analysis, based on the results obtained from the Masson Trichrome stained sections, showed a significantly higher frequency of collagen fragments with EGC areas in Group 1 (EG) than in Groups 2 and 3 (MCT).

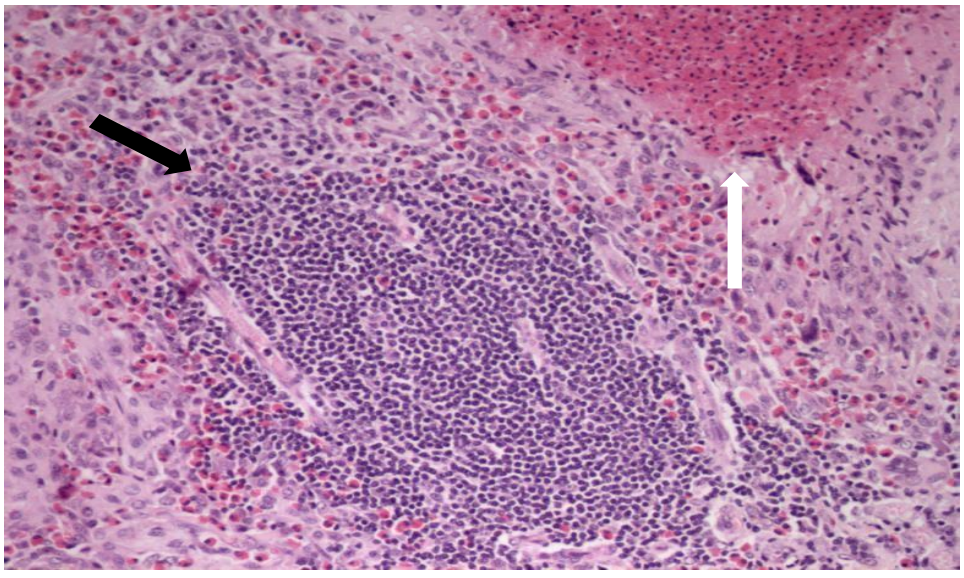
3.4.8 Lymphocyte aggregates

A proportion of cases exhibited aggregates of lymphocytes within the lesions. These were most frequent in Category I lesions and were occasionally present in lesions in Categories II and IV; they were not identified in lesions in either Category III or Category V (Table 15). The aggregates were often relatively large and resembled lymphoid follicles (Figure 20). They were either found intermingled with the EGC or close to the periphery of the lesions.

Table 15: Presence of lymphocyte aggregates in lesions of the five categories

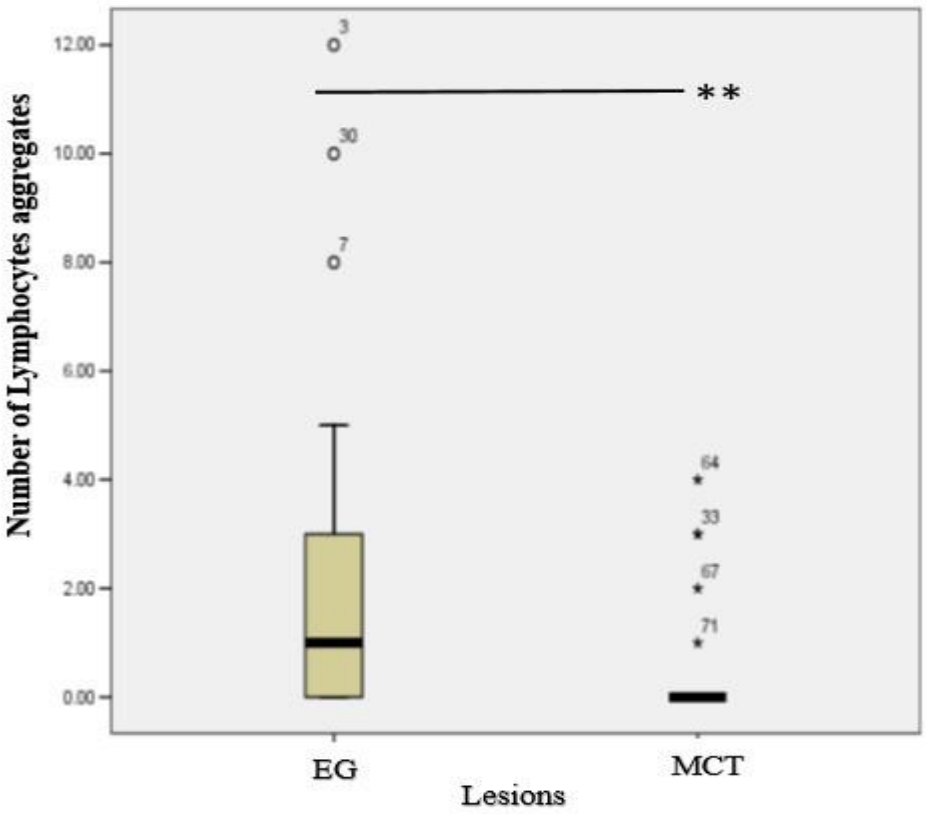
Lymphocyte aggregates, identified in	**No. of total**	**Category**				
		I (n=31)	**II** (n=14)	**III** (n=13)	**IV**(n=23)	**V** (n=14)
HE stain	24/95	61% (19)	14% (2)	0	13% (3)	0

Figure 20: Case No. 06L-4011, Category I. Large lymphocyte aggregate (arrow) close to an EGC area (white arrow). HE stain, 200x



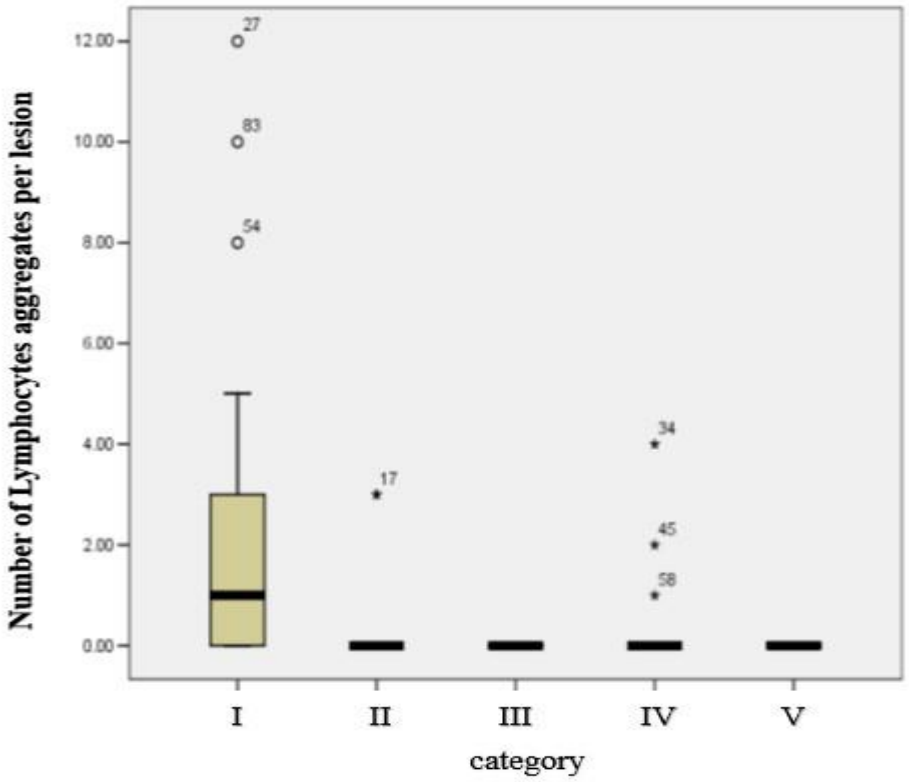
In cases where lymphocyte aggregates were present, these were counted. Statistical analysis showed that Category I lesions (Group 1, EG) contained significantly more lymphocyte aggregates than all the other lesions (Groups 2 and 3 (MCT), Categories II-V) (Figures 21 and 22).

Figure 21: Box and whisker plots to compare the number of lymphocyte aggregates in EG (Group 1) and MCT (Groups 2 and 3).



**=P<0.01. Statistical test (Mann-Whitney U) and the numbers representing outliers.

Figure 22: Box and whisker plots to compare the number of lymphocyte aggregates in EG (Category I) and in all other lesions (Categories II-V).



Statistical test (Mann-Whitney U) and the numbers representing outliers.

3.4.9 Infiltrating viable eosinophils in MCA

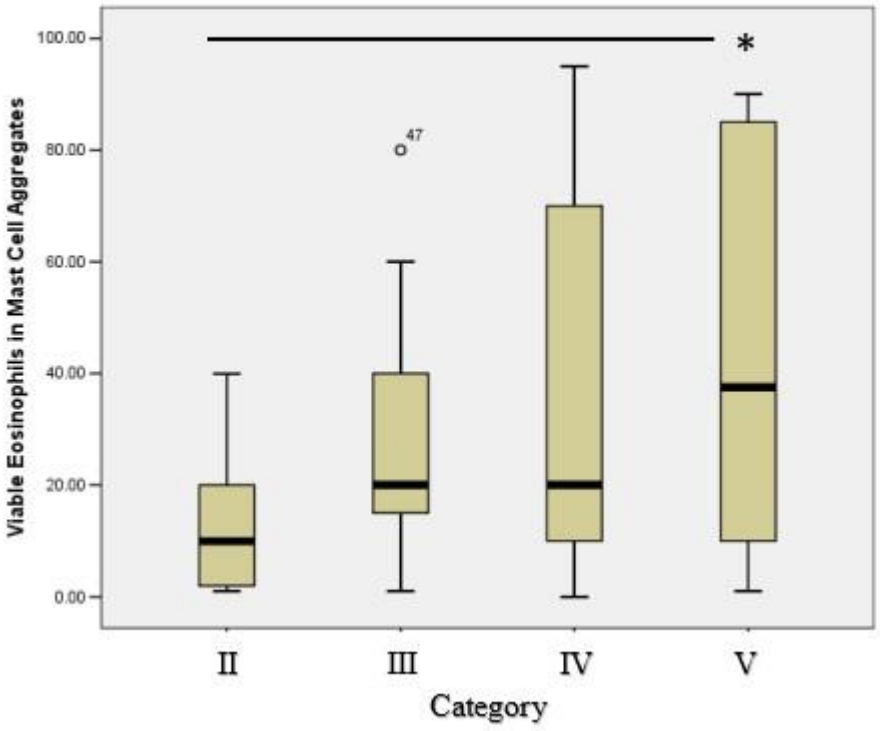
Mast cell aggregates (MCA) were seen in Categories II to V (i.e. in MCT). All MCA contained viable eosinophils in different proportions. The percentage of area occupied by viable eosinophils measured in representative 10 HPFs within MCA increased from an average of only 15% in Category II lesions to 45% in Category V lesions (Table 16). There was, as the table also notes, a gradual increase from Categories II to V. The difference was statistically significant between Category II and V lesions (Figure 23).

Table 16: Average proportion of infiltrating viable eosinophils in MCA in MCT (Categories II-V)

Proportion of infiltrating viable eosinophils in MCA, identified in	**No. of total**	**Category**			
		II (n=14)	**III** (n=13)	**IV** (n=23)	**V** (n=14)
HE stain	64/64	15%	30%	35%	45%

Based on the percentage of area occupied by viable eosinophils measured in 10 representative HPFs within MCA.

Figure 23: Box and whisker plots to illustrate the difference in the proportion of viable eosinophils in MCA in MCT (Categories II-V)



Based on the percentage of area occupied by viable eosinophils measured in 10 representative HPFs within MCA. *=P<0.05. Statistical test (Mann-Whitney U) and the numbers representing outliers.

Further statistical analysis showed a direct correlation between the size of the MCA (see 3.4.1) and the proportion of viable eosinophils in the MCA (P=0.012).

3.4.10 Proliferation of mast cells (mitotic figures) in MCT

All lesions diagnosed as MCT (Categories II-V) were assessed for the proliferative activity of the mast cells, based on the number of mitotic figures (Figure 24). In category II there was only one case in which mitoses were seen. However, these did not exceed 1/10 HPF. In Categories III-V, mitotic figures became progressively more frequent, increasing from 1-2/10 HPF in 3/13 Category III lesions to 8-9/10 HPF in 12/14 Category V lesions (Table 17).

Figure 24: Case No. 06L0445, MCT (Category V). A mitotic figure is evident among neoplastic mast cells (arrow). HE stain, 400x

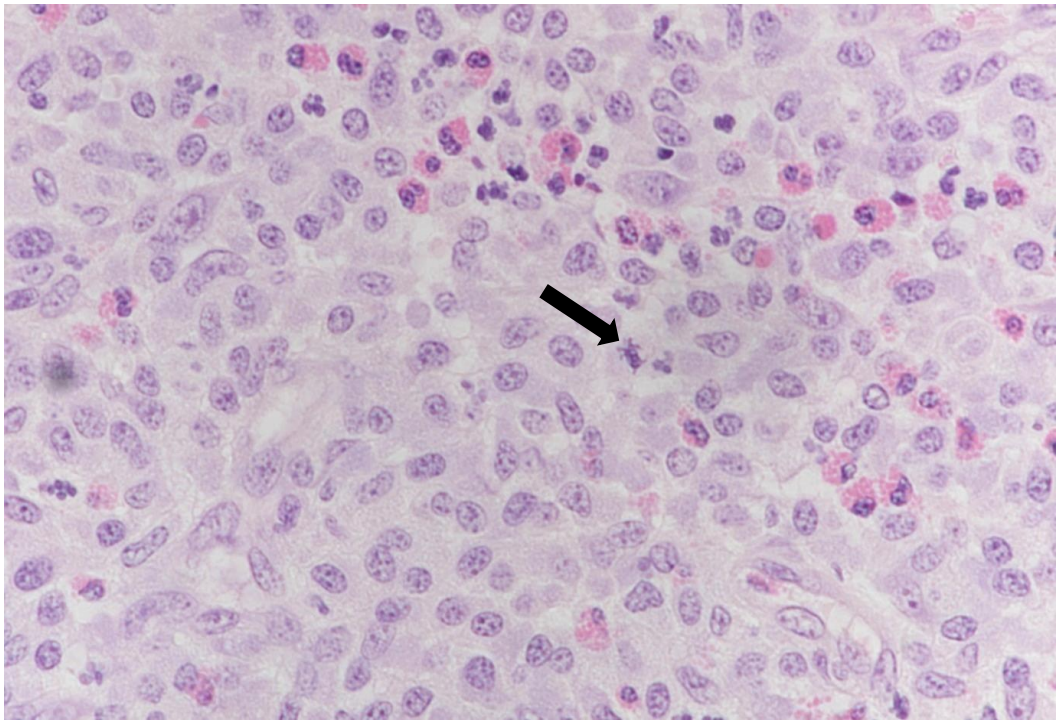


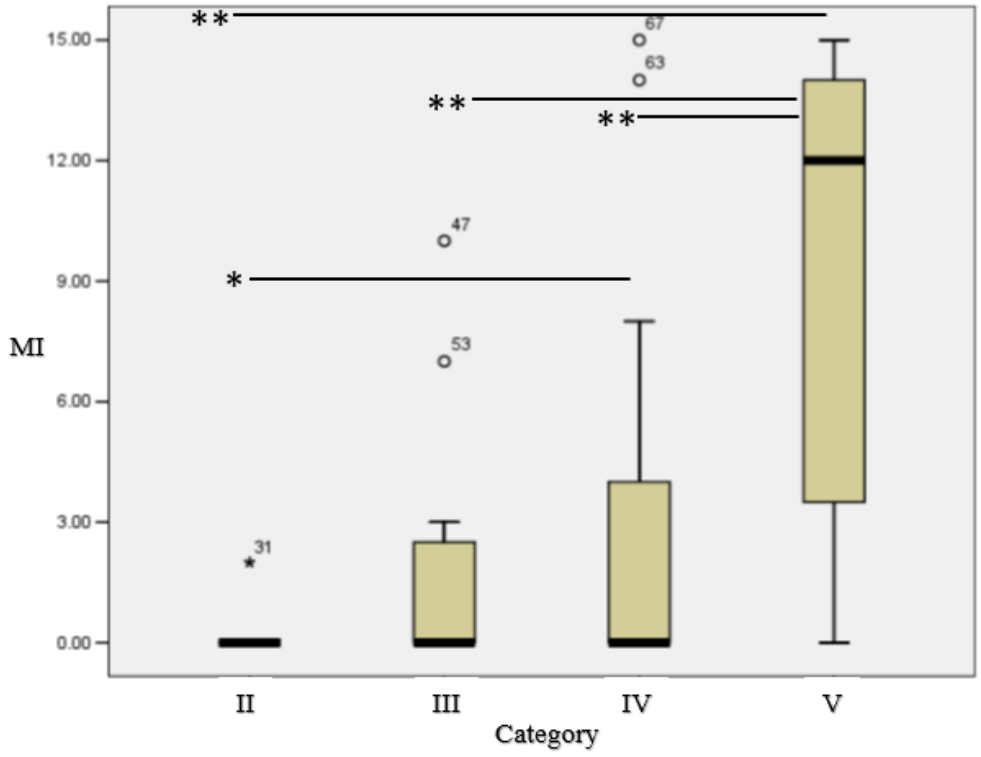
Table 17: Proliferative activity of mast cells in MCT (Categories II-V)

Mitotic cells in MCT, identified in	No. of total	Category			
		II (n=14)	**III** (n=13)	**IV** (n=23)	**V** (n=14)
HE stain	29/64	1 (8%) 1/10 HPF	3 (23%) 1-2/10 HPF	13 (57%) 4/10 HPF	12 (86%) 8-9/10 HPF

The number of mitotic cells per 10 high power fields (HPF) was counted in those cases where mitoses were detected, and an average provided.

The number of mitotic figures progressively increased from Categories II to V, and the difference was significant between Category V and all the others, as well as between Categories IV and II (Figure 25).

Figure 25: Box and whisker plots to illustrate the difference in the number of mitotic figures in MCA between the different categories representing MCT (II-V)

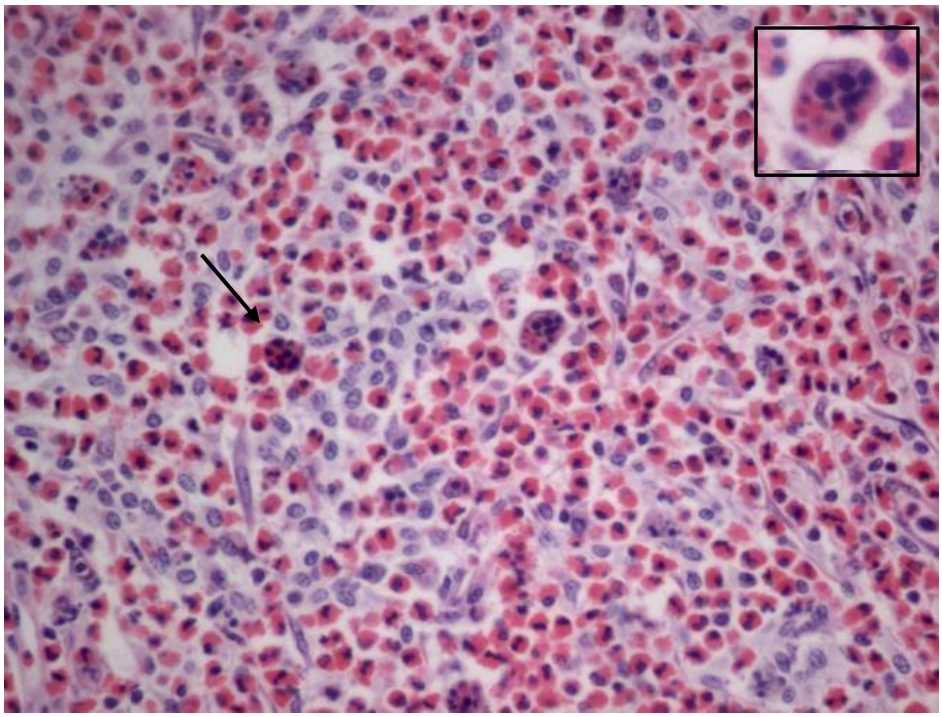


*=P<0.05, **=P<0.01. Statistical test (Mann-Whitney U) and the numbers representing outliers.

3.4.11 Phagocytosis of eosinophils by macrophages in cases with MCA

Phagocytosis of eosinophils by macrophages was observed within MCA in a proportion of MCT in Categories IV and V, where it was seen in 39% (9/23) and 7% (4/14) of lesions, respectively. Phagocytosed eosinophils were generally found within the cytoplasm of the macrophages and occasionally exhibited morphological features of apoptosis, such as shrinking associated with chromatin condensation (Figure 26).

Figure 26: Case No. 15L-4057, Category IV. Several macrophages within the eosinophilrich MCA contain phagocytosed eosinophils (arrows)

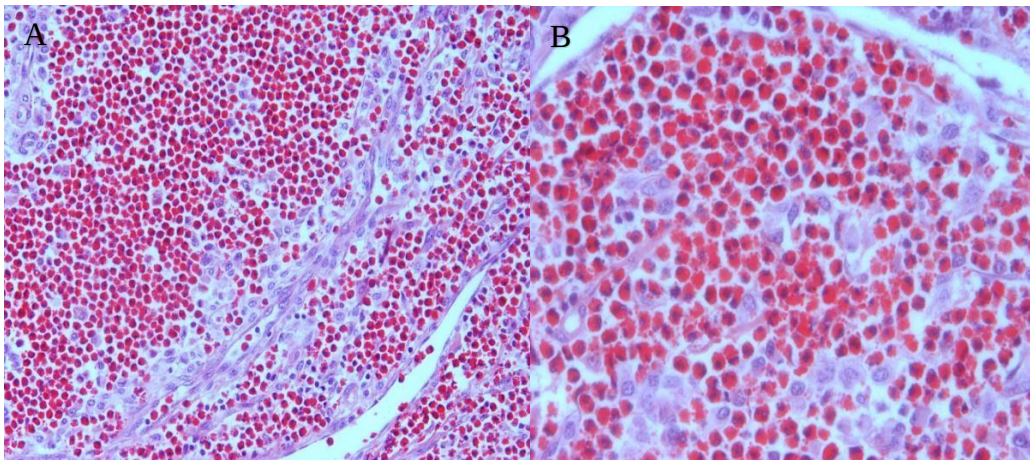


The inset shows a higher magnification of a macrophage with phagocytosed eosinophils that exhibits features of apoptosis. HE stain. 400x and 1000x.

3.4.12 Further analysis of eosinophils and EGC in Lendrum's-stained sections

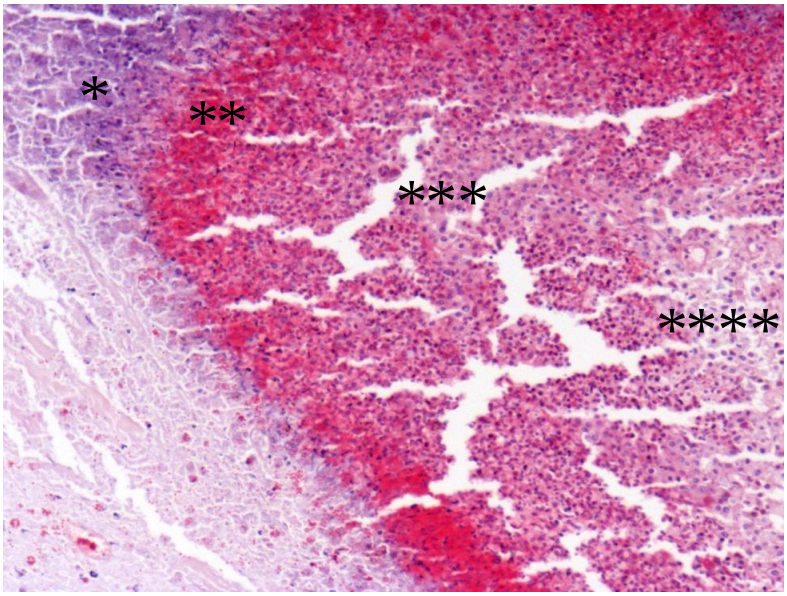
The Lendrum's stain highlighted the granules of eosinophils; they appeared bright red (Figure 27).

Figure 27: Case No. 15L-4057, Category IV. The cytoplasmic granules of eosinophils stain bright red. Lendrum's stain. A, 40x; B, 200x



However, the Lendrum's stain also highlighed areas within the EGC that exhibited different distinct staining intensities; these were categorised as "intense red" (the actual stain of viable eosinophils), "purple", "pale red" and "colourless" (Figure 28).

Figure 28: Case No.07L-2956, category III. The ECG exhibits areas with different staining intensities.



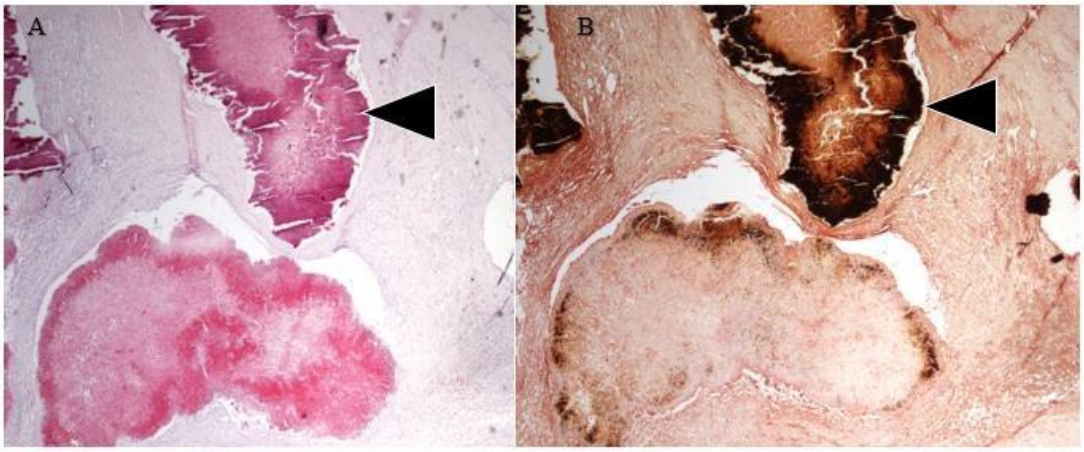
* = purple; ** = intense red; *** = pale red; **** = colourless. Lendrum's stain. 100x.

The evaluation of all the lesions with EGC areas (Categories I-IV) identified these areas to be arranged in irregular concentric rings, with a colourless centre, surrounded by a pale and then intense red zone and separated from other tissue by a purple outer rim. It is evident that the internal part of EGC does not have a regular centrifugal development, but an "irregular onion ring" arrangement, according to the difference in staining as highlighted by Lendrum's stain (Figure 29).

Figure 29: Case No.06L1943, category III. "Onion ring"-like arrangement of zones with different staining intensity in an EGC. Lendrum's stain. 40x

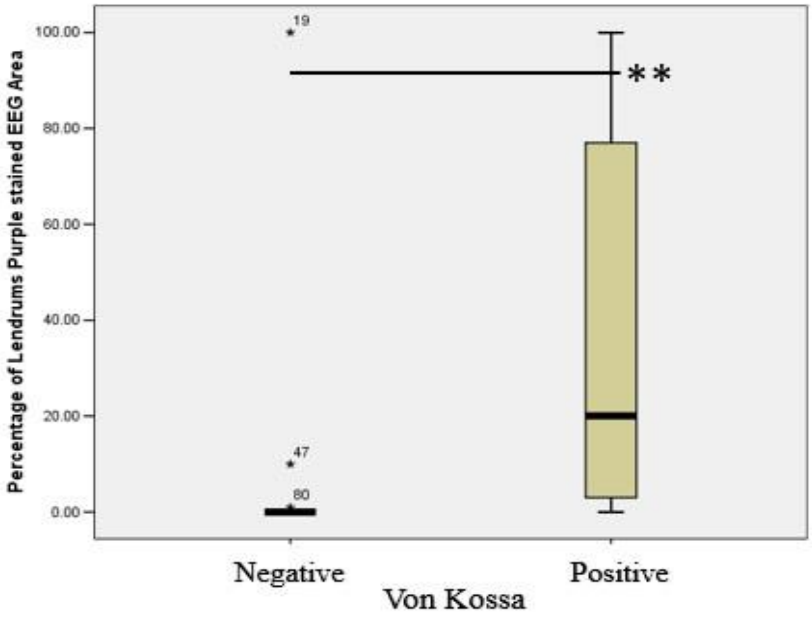
The comparative examination of the Lendrum's stained sections with a consecutive von Kossa-stained section showed that the purple areas in the Lendrum's stain were identical to the von Kossa-positive areas, i.e. represent mineralisation (Figure 30).

Figure 30: Case No.3595, Category III. In the EGC, the purple areas highlighted by the Lendrum's stain (A; arrow) correlates with the von Kossa-positive areas (B; arrow). 40x



The assessment of all lesions stained with both special stains showed a significant correlation between the von Kossa-positive areas and the areas that were purple with the Lendrum's stain. This confirmed that both detected areas had mineralisation (Figure 31).

Figure 31: Box and whisker plots to illustrate the difference in the percentage of Lendrum's purple stained areas between cases exhibiting or not exhibiting von Kossa positive areas of mineralisation in EGC



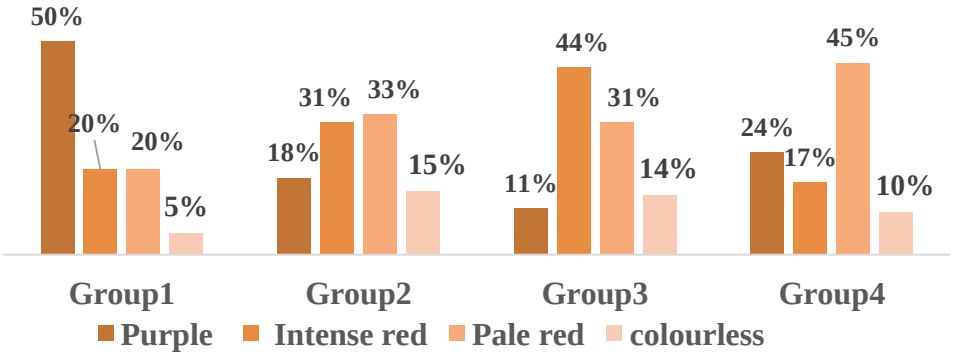
**=P<0.01. Statistical test (Mann-Whitney U) and the numbers representing outliers.

The fading red colour, from intense red through pale red to colourless, was tentatively interpreted as representative of degranulation/degeneration of eosinophils, suggesting the following staining pattern in Lendrum's stained sections:

- Purple = mineralisation
- Intense red = viable eosinophils
- Pale red = early degenerating/degranulating eosinophils
- Colourless = late degenerating/degranulated eosinophils

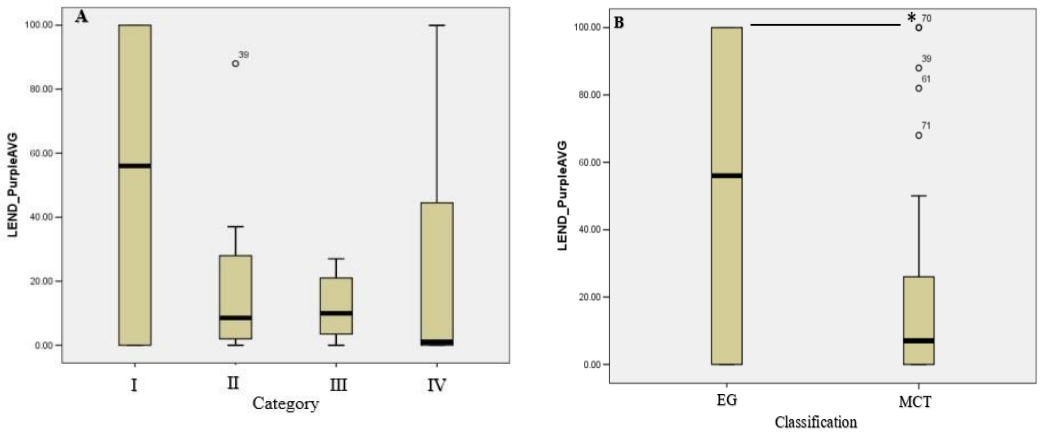
Assessing that the lesions that contained eosinophils (Categories I to IV) for the area within EGC covered by the four different staining patterns confirmed that the larger areas of mineralisation (purple colour) were more abundant within Category I lesions (on average 50% of EGC area). Intense red staining areas (representing aggregates of viable eosinophils) were most abundant in Categories II and III (31% and 44% of EGC area), whereas the pale red areas that were indicative of accumulations of early degranulating eosinophils increased from Category I (20%) over Categories II and III (33% and 31%) to Category IV (45%). Areas composed of degenerated (i.e. late degenerating) eosinophils were least abundant in Category I (5%), and accounted for 15%/14% in Categories II and III, and for 10% of the area in Category IV (Figure 29). The findings confirm that mineralisation is most pronounced in pure EG (Category I), and suggests that EGC areas in Categories II and III contain more viable eosinophils than the pure EG and EGC of Category IV lesions. Nonetheless, areas with degranulated eosinophils (early stage) became gradually more abundant from Categories II to IV. Interestingly, areas with eosinophils in late degranulation were again most abundant in Categories II and III (Figure 32).

Figure 32: Comparison of the proportion of EGC areas with the different staining intensities after the Lendrum's stain (N=81)



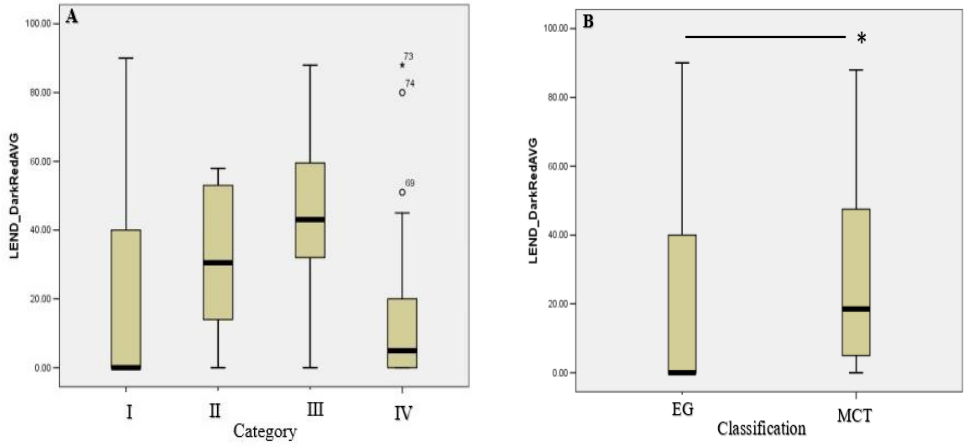
The statistical analysis confirmed the above findings and highlighted that mineralisation was significantly more abundant in pure EG (Category I) than in MCT with EGC (Categories II-IV), while intense red, pale red and colourless areas were significantly more abundant in EGC areas within MCT (Categories II-IV) (Figures 34 to 37).

Figure 33: Box and whisker plots to illustrate the difference in percentage of Lendrum's purple stained areas in EGC within categories (A) and EG (Group 1) vs. MCT (Groups 2 and 3) (B)



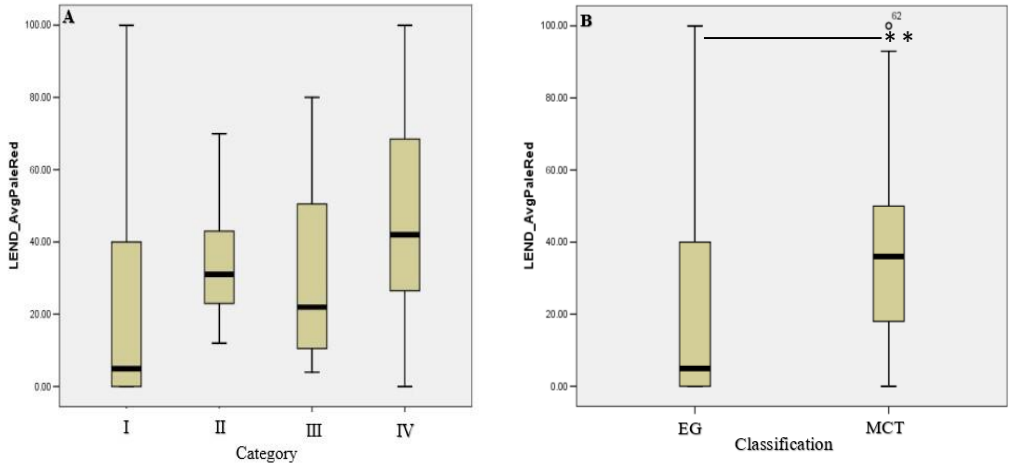
*=P<0.05. Statistical test (Mann-Whitney U) and the numbers representing outliers.

Figure 34: Box and whisker plots to illustrate the difference in percentage of Lendrum's dark red stained areas in EGC within categories (A) and EG (Group 1) vs. MCT (Groups 2 and 3) (B)



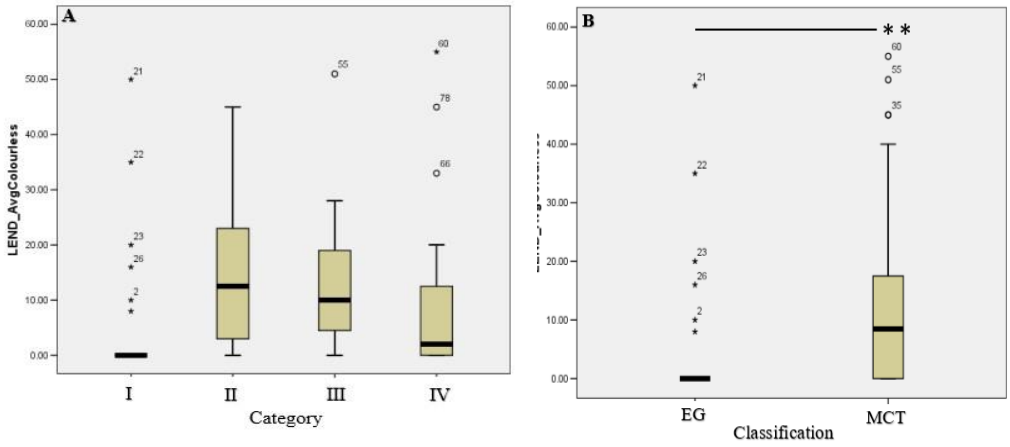
*=P<0.05. Statistical test (Mann-Whitney U) and the numbers representing outliers.

Figure 35: Box and whisker plots to illustrate the difference in percentage of Lendrum's pale red stained areas in EGC within categories (A) and EG (Group 1) vs. MCT (Groups 2 and 3) (B)



**=P<0.01. Statistical test (Mann-Whitney U) and the numbers representing outliers.

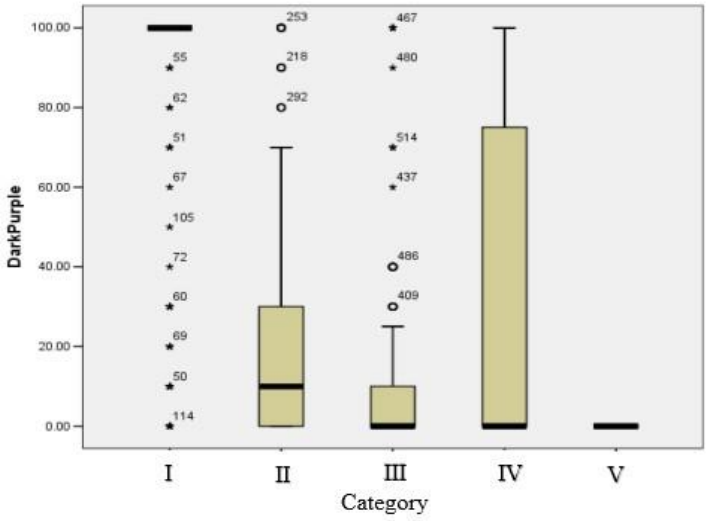
Figure 36: Box and whisker plots to illustrate the difference in percentage of Lendrum's colourless areas in EGC within categories (A) and EG (Group 1) vs. MCT (Groups 2 and 3) (B)



**=P<0.01. Statistical test (Mann-Whitney U) and the numbers representing outliers.

When all the 724 individual EGC areas that were found in all the lesions were examined, the Lendrum's stain results were further confirmed. There was a strong trend to associate dark purple areas (mineralisation) with Category I lesions (EG; Figure 37).

Figure 37: Box and whisker plots to illustrate the difference in the percentage of Lendrum's purple areas in EGC (n=724) among categories



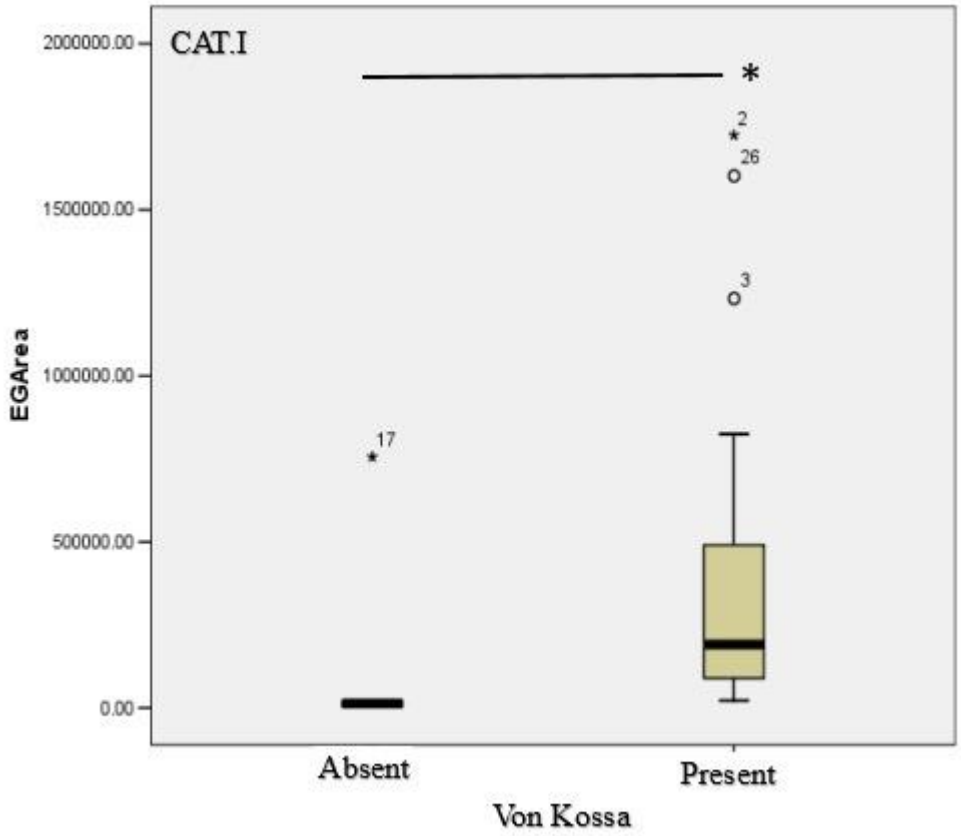
*=P<0.05. Statistical test (Mann-Whitney U) and the numbers representing outliers.

3.4.13 Correlation of lesion size, mineralisation and possible age of the lesion

The above results led to the formation of the following hypothesis: that a larger EGC lesion would be older and therefore more prone to dystrophic mineralisation.

However, correlating the size of and the presence of mineralisation in EGC in the von Kossa-stained sections failed to find a general association between size and mineralisation of EGC areas (Figure 17). Interestingly, this was different when Category I lesions were examined on their own. Here, a correlation between size and mineralisation was evident for EGC areas (Figure 38).

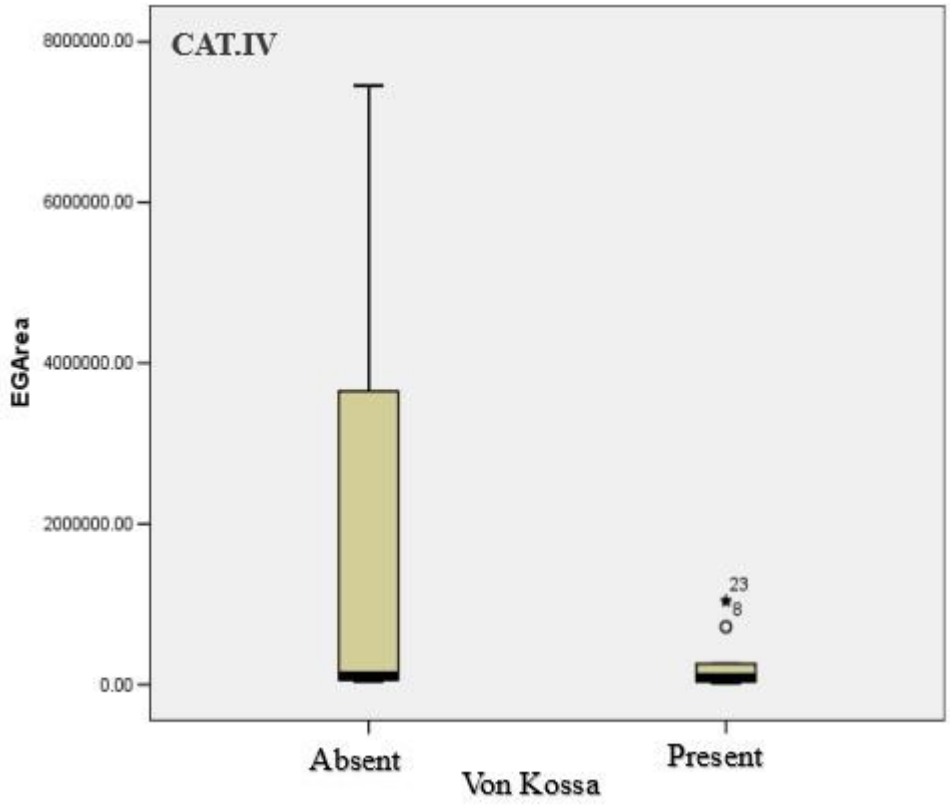
Figure 38: Box and whisker plots to illustrate the difference in EGC area (pixels) between cases of category I with Von Kossa positive or negative stain.



*=P<0.05. Statistical test (Mann-Whitney U) and the numbers representing outliers.

In the other categories there was no significant difference. In Category IV lesions, a trend towards a negative correlation was identified instead (Figure 39).

Figure 39: Box and whisker plots to illustrate the difference in EGC area between cases of Category IV with or without Von Kossa positive stain in EGC



*=P<0.05. Statistical test (Mann-Whitney U) and the numbers representing outliers.

3.5 Immunohistochemical detection of T cells (CD3+), B cells (CD79a+) and macrophages (Iba1+) in the different categories and groups

3.5.1 T cells

T cells (CD3-positive) were found within lesions of all categories. They were found scattered throughout the lesions and associated predominantly with EGC areas (Figure 40); they were also identified scattered throughout the MCA (Figure 41).

Figure 40: Case No.13L-3963, Category I. CD3 positive T cells infiltrating the surrounds of an area of EGC (asterisk). Immunoperoxidase, 200x

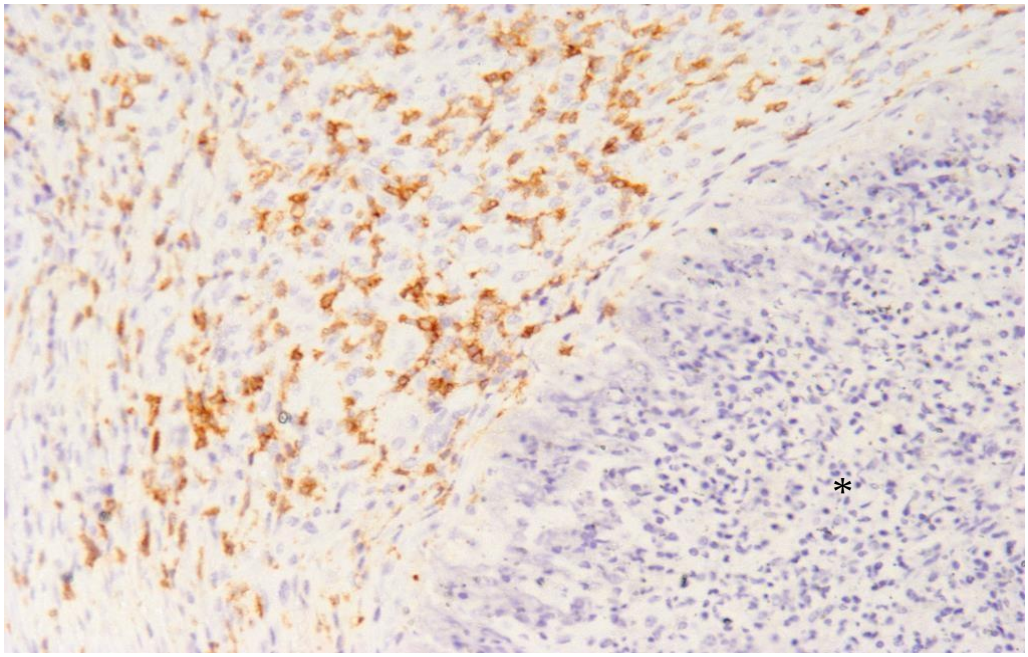
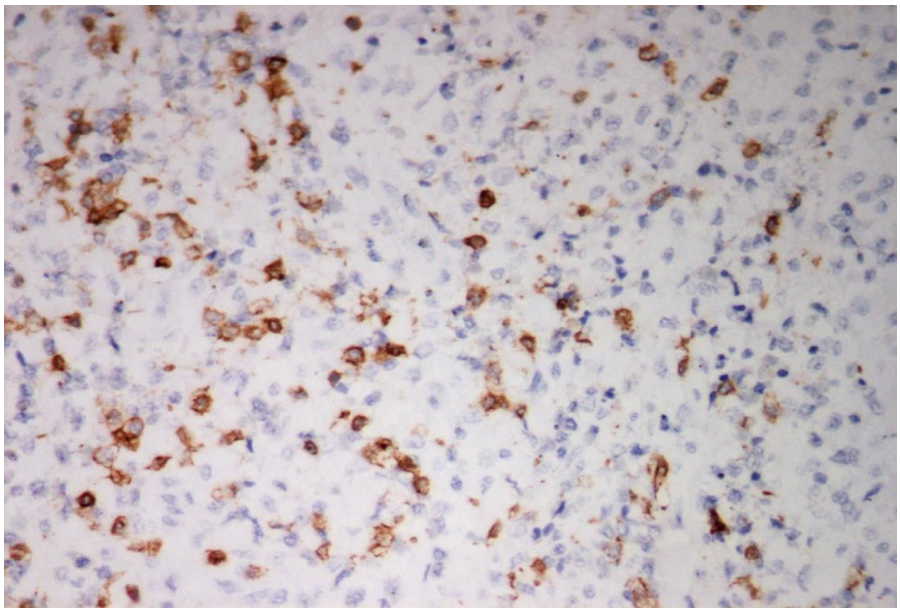
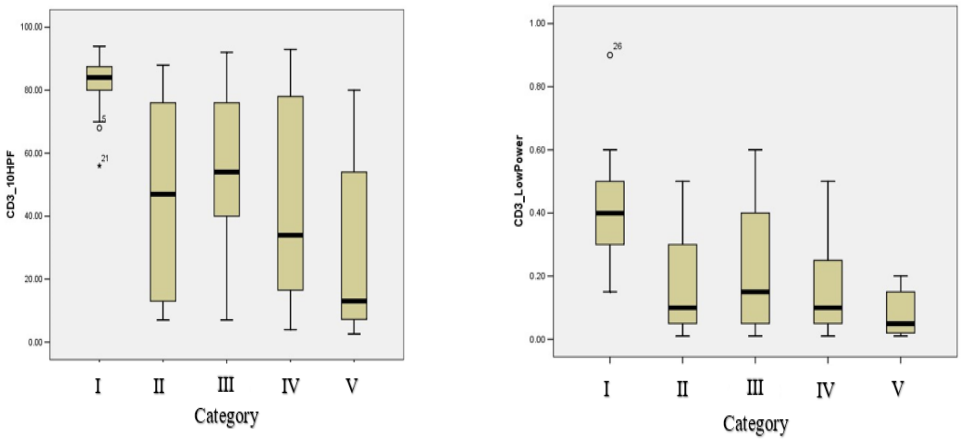


Figure 41: Case No. 09L3044, Category III. CD3 positive T cells infiltrating a MCA area. Immunoperoxidase, 400x



There was an increase in CD3 positive cell density infiltrating Category I lesions compared to Categories II-V whether ollowing examination over 10 HPFs or assessed as an overall percentage of lesion area occupied by CD3 positive cells; however, the difference between categories were not significant (Figure 42).

Figure 42: Box and whisker plots to illustrate the difference in CD3 positive cell density among categories evaluated in multiple HPFs (A) or at low power (B)

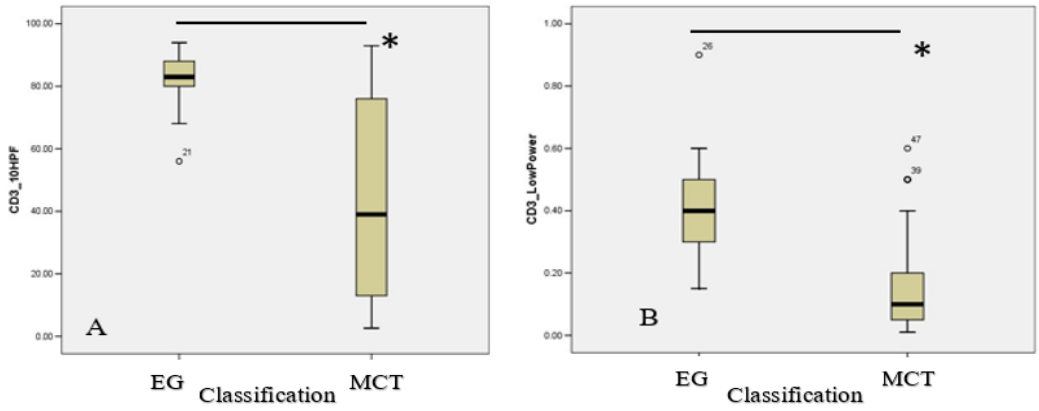


A B

Statistical test (Mann-Whitney U) and the numbers representing outliers.

Comparing T cell (CD3-positive) density between EG (Group 1) and MCT (Groups 2 and 3), a significant difference was found, with EG exhibiting an increased CD3 positive cell density (Figure 43).

Figure 43: Box and whisker plots to illustrate the difference in CD3 positive cell density between EG and MCT evaluated in multiple HPFs (A) or at low power (B)

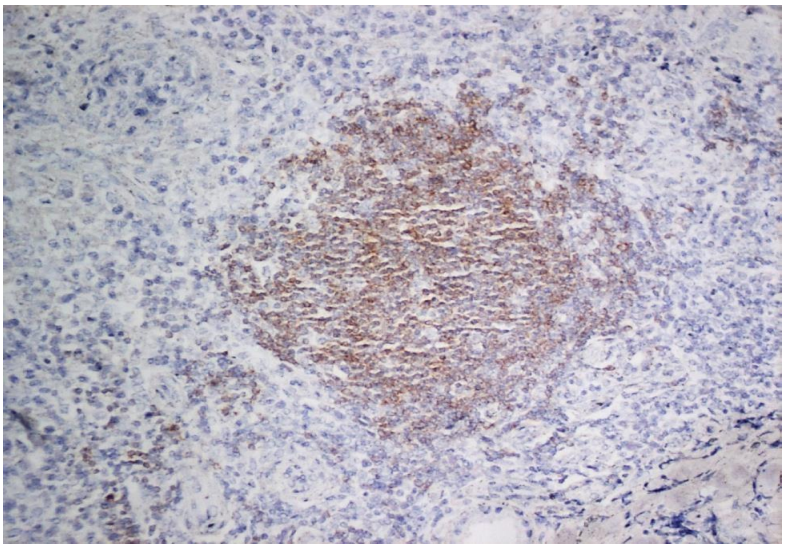


*=P<0.05. Statistical test (Mann-Whitney U) and the numbers representing outliers.

3.5.2 B cells

B cells (CD79a- positive) were only occasionally seen in association with the lesions. They were almost always clustered, forming follicle-like structures, with overlapping that was identified morphologically as lymphocyte aggregates (Figure 44). These were either found intermingled with the lesions or close to their periphery.

Figure 44: Case No. 11L-2349, Category I. CD79a positive B cells forming a discrete, follicle-like aggregate. Immunoperoxidase, 200x



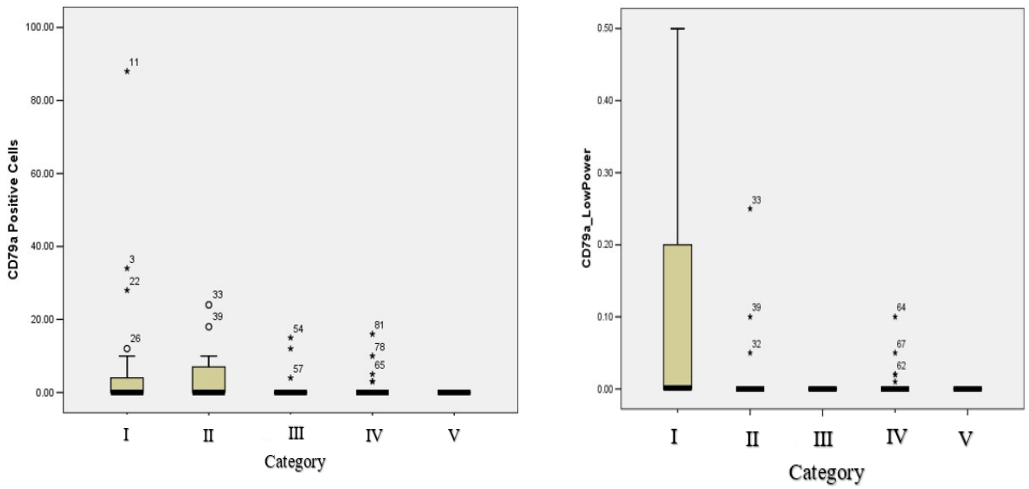
Considering cases with at least one CD79a positive cell, the vast majority of cases was in Category I (Table 18).

Table 18: Distribution of cases exhibiting at least one CD79a positive cell among categories

CD79a positive cells in the cases, identified in	**No. of total**	**Percentage and numbers of cases in the indicated category**				
		I (n=31)	**II** (n=14)	**III** (n=13)	**IV** (n=23)	**V** (n=14)
CD79a IHC stain	23/95	48% (15)	21.4% (3)	0	21% (5)	0

When these cells were quantified there was a higher density of CD79a positive cells in Category I compared to the other categories (Fig.3.42). Due to the low number of cases exhibiting CD79a positive cells at all, a significant difference was only detected comparing EG (Group 1) against MCT (Group 2) (Figure 46).

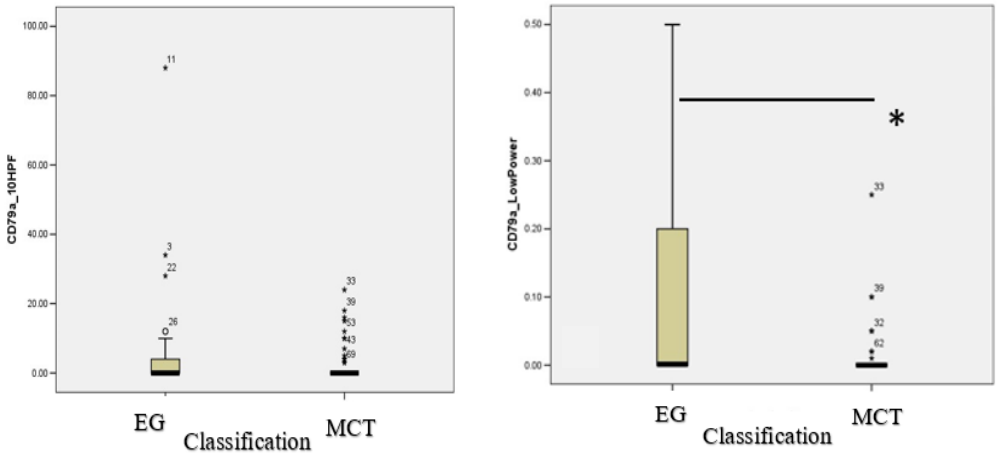
Figure 45: Box and whisker plots to illustrate the difference in CD79a positive cell density among categories evaluated in multiple HPFs (A) or at low power (B)



A B

Statistical test (Mann-Whitney U) and the numbers representing outliers.

Figure 46: Box and whisker plots to illustrate the difference in CD79 positive cell density among EG and MCT evaluated in multiple HPFs (A) or at low power (B)



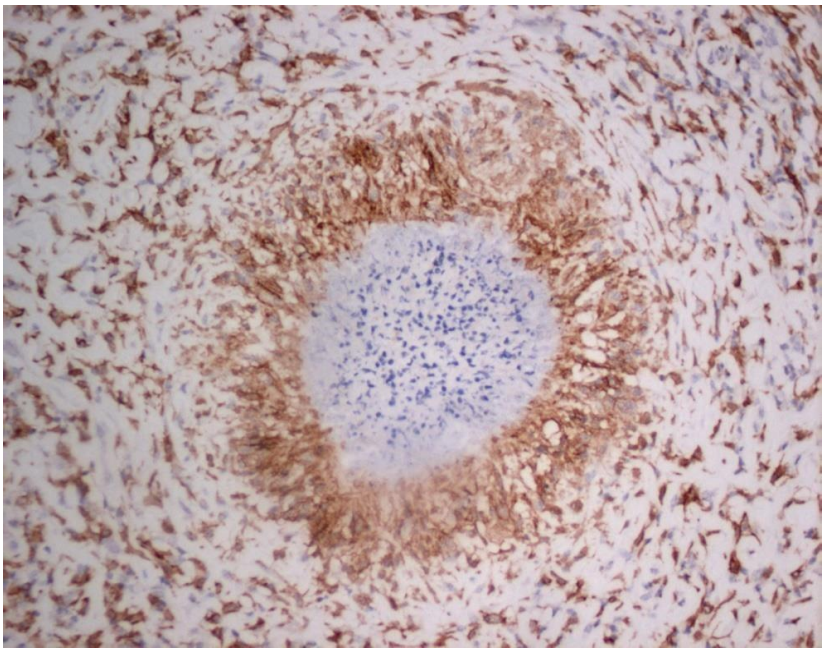
A B

*=P<0.05. Statistical test (Mann-Whitney U) and the numbers representing outliers.

3.5.3 Macrophages

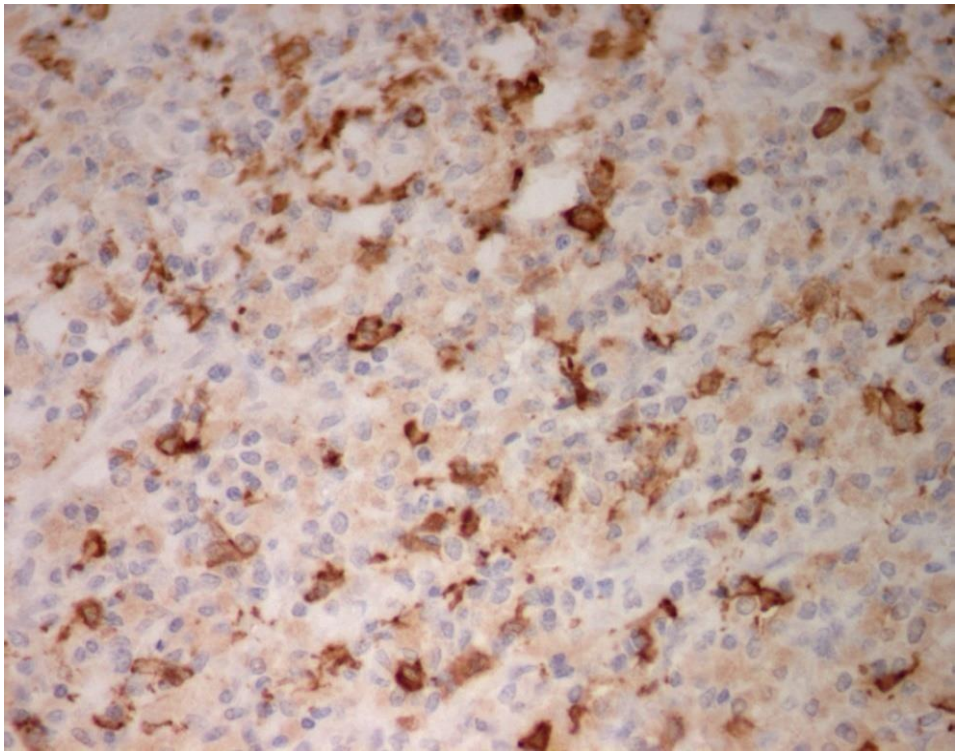
Iba1-positive cells were large and pleomorphic, and morphologically consistent with macrophages. Iba1 was consistently expressed by epithelioid macrophages forming EGC and surrounding degenerated eosinophils, in addition to more random, scattered, and more peripheral macrophages (Figure 47).

Figure 47: Case No.11L-3991, Category I. Iba1 positive macrophages palisading around the core of an EGC, and are also found scattered as individual cells in its periphery. Immunoperoxidase, 200x



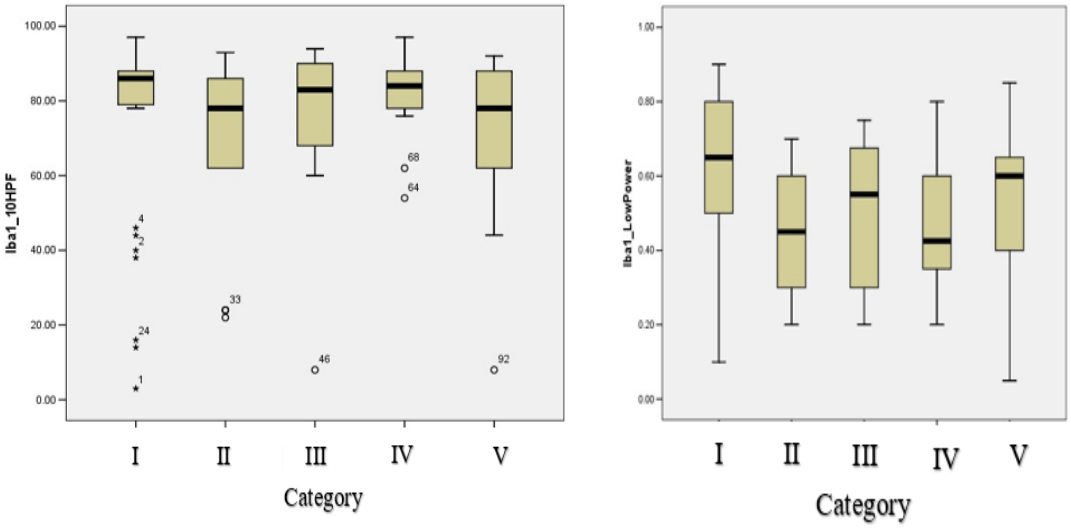
Iba1- positive macrophages were also consistently detected infiltrating MCA in association with mast cells (Figure 48). A variable mild positive reaction was also detected in the cytoplasm of mast cells.

Figure 48: Case No.07L-3335, Category IV. Iba1- positive macrophages scattered in between mast cells in a MCA area. Immunoperoxidase, 200x



Comparing the different categories as well as EG against MCA, there was no difference in the number of Iba1-positive cells (Figure 49), except for a higher density in EG compared to MCT when estimated at low power (Figure 50).

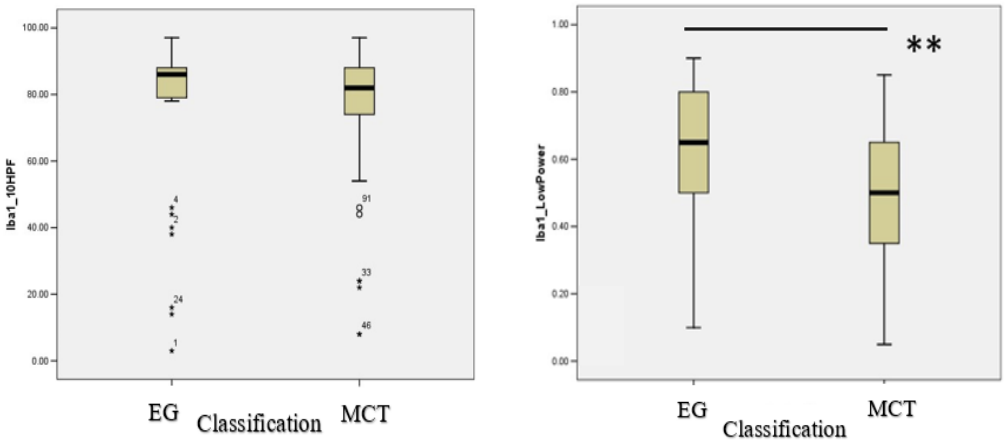
Figure 49: Box and whisker plots to illustrate the differences in Iba1 positive cell density among categories evaluated in multiple HPFs (A) or at low power (B)



A B

*=P<0.05. Statistical test (Mann-Whitney U) and the numbers representing outliers.

Figure 50: Box and whisker plots to illustrate the difference in Iba1 positive cell density among EG and MCT evaluated in multiple HPFs (A) or at low power (B)



A B

*=P<0.01. Statistical test (Mann-Whitney U) and the numbers representing outliers.

3.6 Cytokine and chemokine expression in EG and MCT

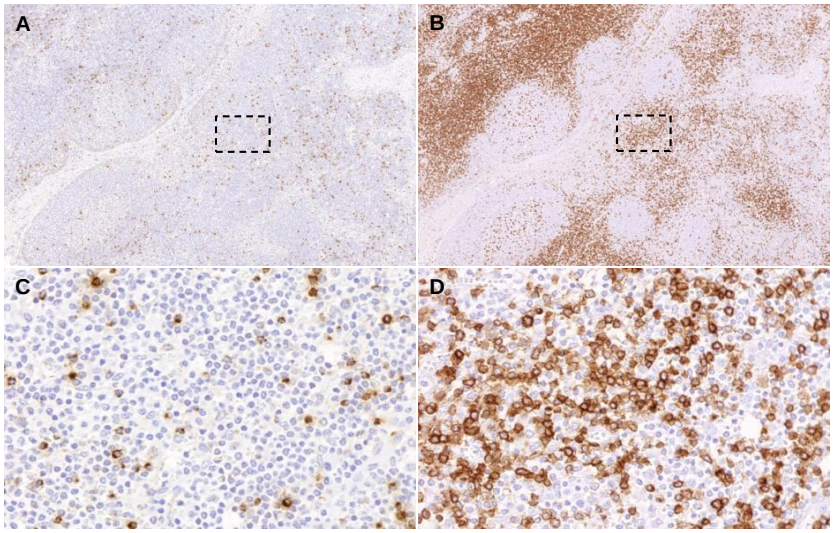
This part of the study aimed to reveal a range of mechanistic and pathogenetic aspects of both MCT and EG, using an *in situ* approach based on the differential expression of a range of cytokines and chemokines that can be expected to influence the interplay between eosinophils, and other relevant cell populations in both entities (i.e. mast cells, macrophages and lymphocytes).

While a commercial antibody was available for RANTES/CCL5, these lacked for the remaining markers of interest. Accordingly, an mRNA-ISH (RNAscope® technology) approach was taken, using a representative cohort of 10 cases (Appendix II –section A).

3.6.1 Expression of RANTES/CCL5 (IHC)

RANTES/CCL5 is known to be expressed in T cells, MPH, EOs and MCs, and to exert mediating effects to all these cells. We tested the antibody in the unaltered lymph node of a horse, and compared the staining with a consecutive section stained for the expression of the pan-T-cell marker CD3. The results indicate that RANTES is expressed in a minor subpopulation of T cells (CD3-positive) (Figure 51).

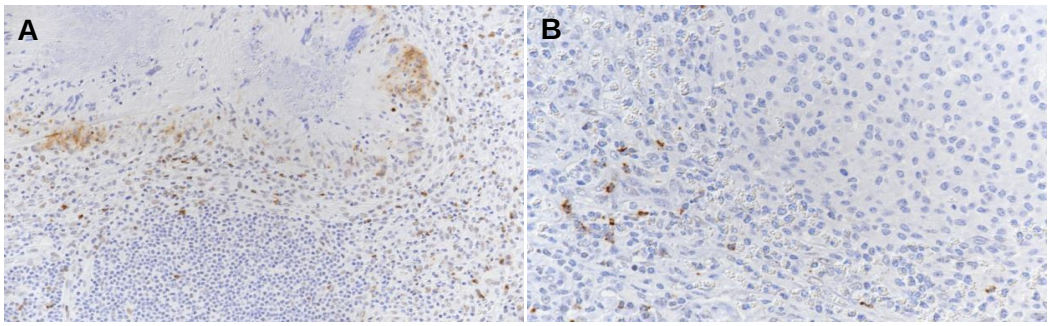
Figure 51: Comparison of RANTES (A, C) vs CD3 (B, D) expression in a control lymph node



Whereas abundant T cells comprise the paracortical T cell zones, only a minor subpopulation of these cells express RANTES (C). C and D represent higher magnifications of the insets of pictures A and B, respectively. IHC, immune-peroxidase, A, B 50x; C, D, 400x.

Indeed, RANTES expression was also observed mainly in lymphocytes in both types of lesions. However, in EG, as well as in palisading macrophages were found to be positive (Figure 52).

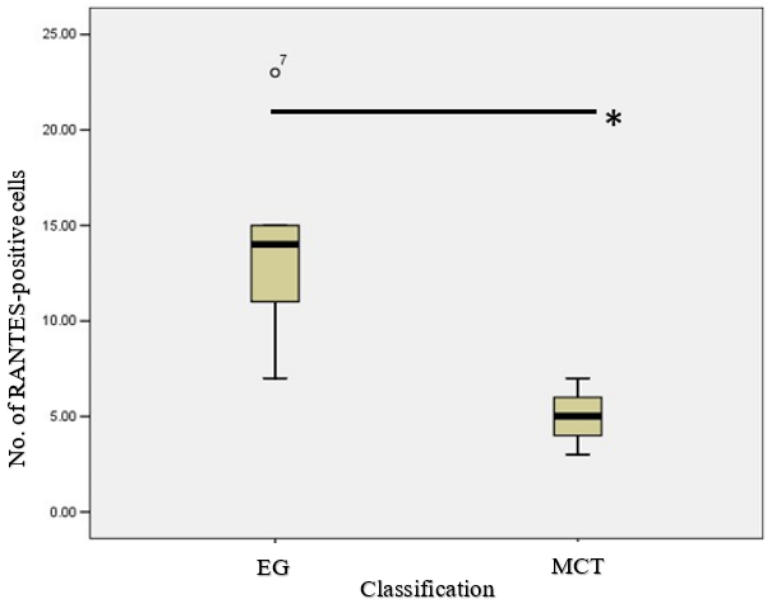
Figure 52: RANTES. (A) Case No. 09L-3297, Category I. (B) Case No. 06L1257 (MCT-4).



(A) RANTES expression was observed mainly in lymphocytes and in palisading macrophages. 200x. (B) MCT. RANTES expression is restricted to some scattered lymphocytes. 400x. IHC immunoperoxidase.

RANTES-positive cells were significantly more numerous in EG (Category I) than in MCT (Categories III-IV) (Figure 53).

Figure 53: Box and whisker plots to illustrate the difference in the number of RANTES positive cells in EG (n=5) and MCT (n=5).

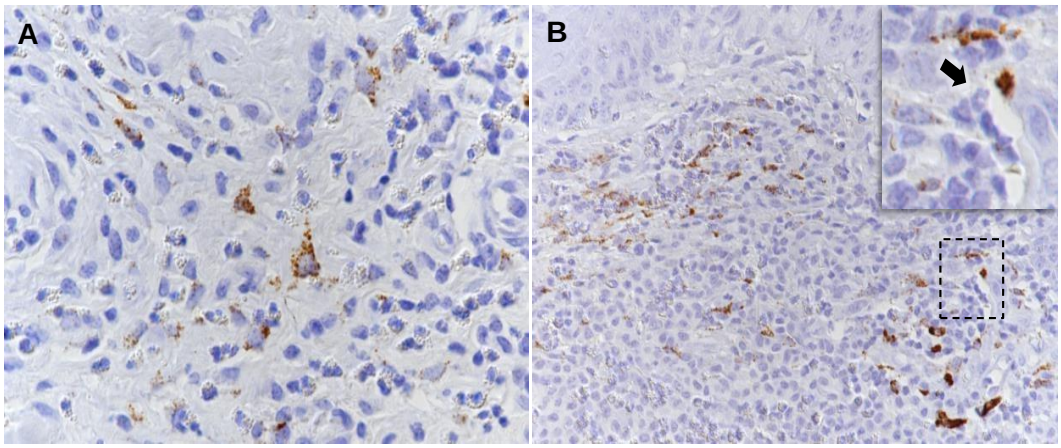


*=P<0.05. Statistical test (Mann-Whitney U) and the numbers representing outliers.

3.6.2 Expression of Eotaxin/CCL11

Eotaxin was generally expressed intensely in lymphocytes, macrophages and fibroblastoid cells in EG (Figure 54A). In contrast, MCT only very rarely exhibitedpositive cells, and only in some ulcerated lesions with infiltrating neutrophils, where focal eotaxin expression was seen in macrophages and fibroblastoid cells (not included in the cell counts). The MCA themselves always remained negative (Figure 54B).

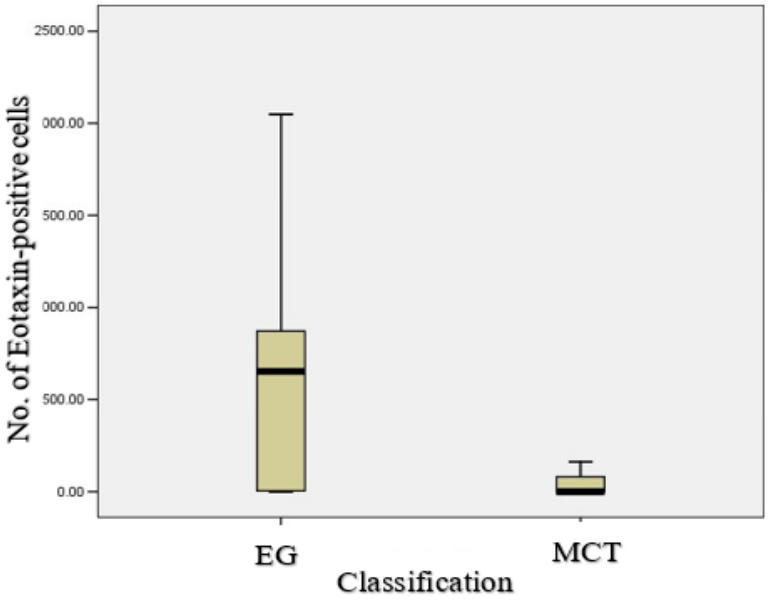
Figure 54: Eotaxin. (A) Case No. 13L-4160, Category I. (B) Case No. 11L-4484, Category IV.



(A) Eotaxin expression was observed in lymphocytes, macrophages and fibroblastoid cells. (B) Focal eotaxin expression in macrophages and fibroblastoid cells in an MCT with infiltrating neutrophils (inset). The rest of MCA remain negative. mRNA-ISH, A 600x, B 400x and 1000x (inset).

The eotaxin expression pattern suggests a major role of this chemokine in EG, but not in MCT. However, this difference was not statistically significant (Figure 55).

Figure 55: Box and whisker plots to illustrate the difference in the average number of eotaxin positive cells in EG (n=5) and MCT (n=5)

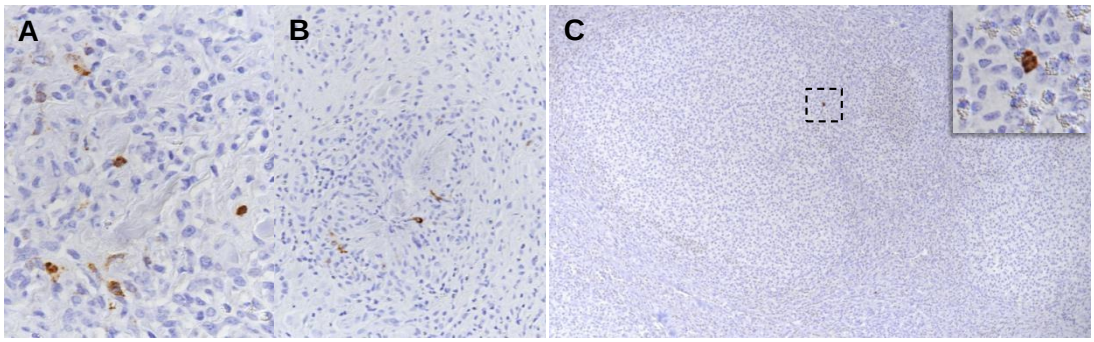


Statistical test (Mann-Whitney U).

3.6.3 Expression of MIPα/CCL3

MIPα expression was detected in scattered lymphocytes, macrophages and some palisading macrophages in EG (Figure 56, A, B). In MCT, a very weak MIPα mRNA signal was detected in some lymphocytes (Figure 56, C)

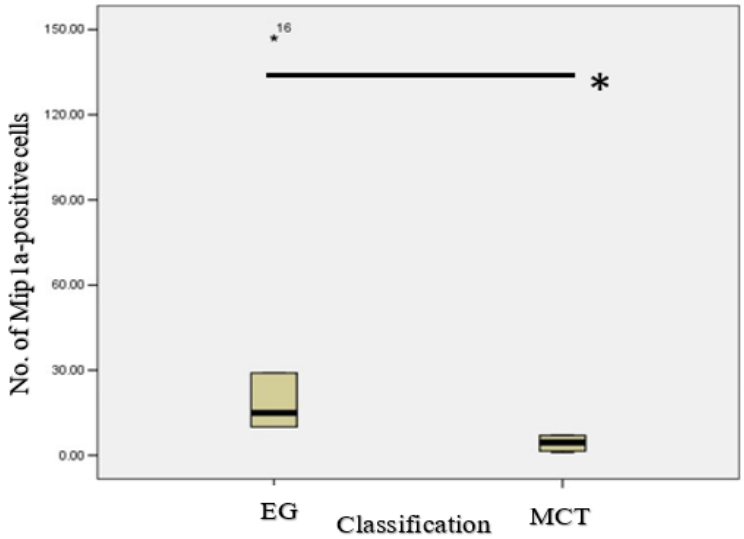
Figure 56: MIPα. (A, B) Case No. 13L-4160, category I. (C) Case No. 06L-1257, category IV.



MIPα. (A, B) MIPα expression was detected in scattered lymphocytes and macrophages (A) and in some palisading histocytes (B) in EG.
(C) Minimal expression of MIPα mRNA was detected in some lymphocytes in MCT.
mRNA-ISH, A 400x, B200x, C100x and 1000x (inset).

In both entities, the number of positive cells was very low. Nonetheless, the expression of MIPα was more intense in EG compared to MCT (Figure 57).

Figure 57: Box and whisker plots to illustrate the difference in MIPα positive cells between EG (n=5) and MCT (n=4)

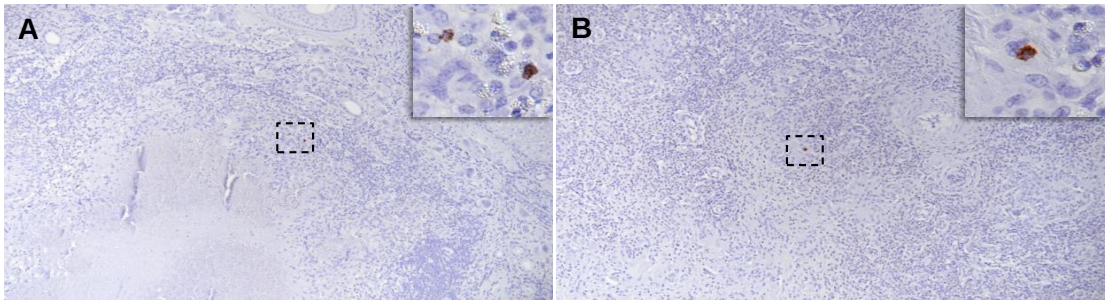


*=P<0.05. Statistical test (Mann-Whitney U) and the number representing outlier.

3.6.4 Expression of interleukin 4 (IL-4)

IL-4 mRNA was detected in scattered lymphocytes in EG (Figure 3.55 A, B). Interestingly, no expression was seen in any cells in any of the MCT.

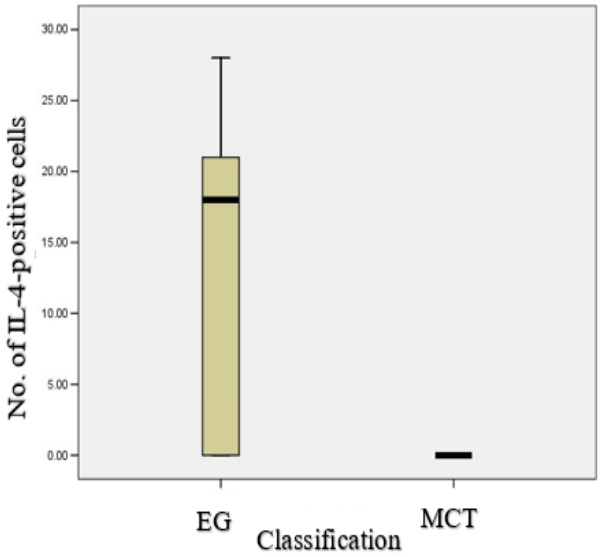
Figure 58: IL-4. (A) Case No. 11L-2439, category I. (B) Case no. 13L-4160, category I.



IL-4 expression was detected in very scattered lymphocytes in EG. RNA-ISH, A 100x, B 100x and 1000x (inset).

A minimal expression of IL-4 was observed in this study, and only very few scattered positive cells were detected in cases of EG (Figure 59).

Figure 59: Box and whisker plots to illustrate the difference in IL-4 positive cells between EG (n=5) and MCT (n=3).

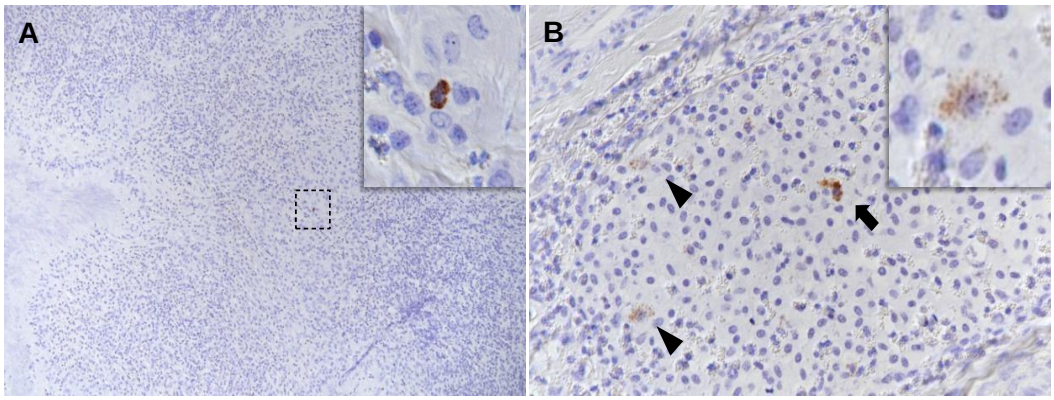


Statistical test (Mann-Whitney U).

3.6.5 Expression of interleukin 5 (IL-5)

In EG, IL-5 expression was restricted to some scattered individual lymphocytes (Figure 60, A). In MCT, a strong IL-5 signal was seen in a few scattered lymphocytes, and a less intense signal (faint dots) in some mast cells (Figure 60, B); overall, the expression was more intense than in EG.

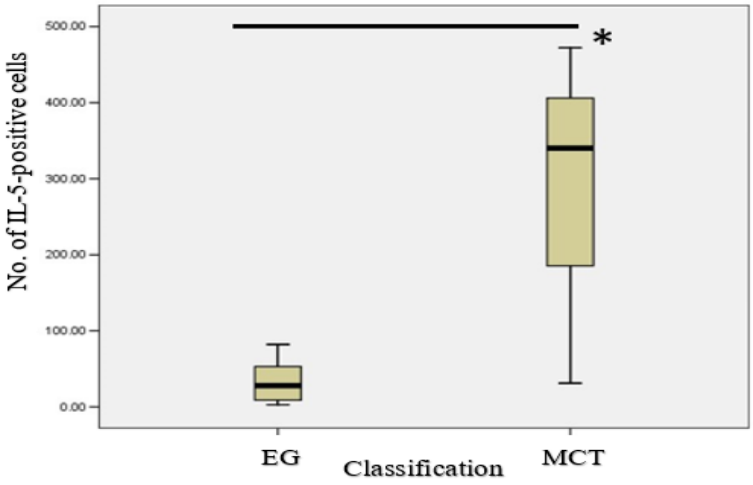
Figure 60: IL-5. (A) Case No. 09L-3297, category I. (B) Case No. 15L-1571, category III



(A) IL-5 expression in EG was restricted to some scattered lymphocytes (inset). (B) In MCT, IL-5 showed a strong reaction in a few scattered lymphocytes (arrow), and a less intense signal (faint dots) in some mast cells (arrowheads, inset). mRNA-ISH, A 100x, B 400x and 1000x (inset).

IL-5 expression was seen in significantly higher numbers of cells in MCT than in EG (Figure 61).

Figure 61: Box and whisker plots to illustrate the difference in IL-5 positive cells between EG (n=5) and MCT (n=5)



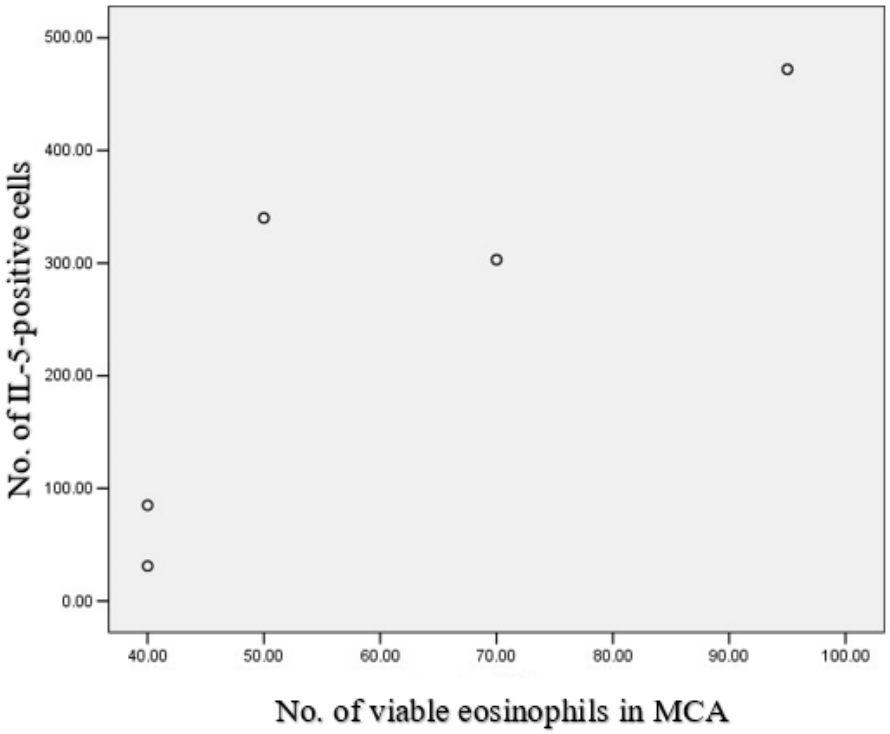
*=P<0.05. Statistical test (Mann-Whitney U) and the numbers representing outliers.

Interestingly, a strong relation between the density of viable eosinophils (% of the area with eosinophils in HPFs) and IL-5 expression was observed within MCA (Figure 62).

Figure 62: Correlation between IL-5 mRNA-positive cells and viable eosinophils in MCA (n=5)

Correlations

		Eos_in_MCAggre	IL5_Quant
Eos_in_MCAggre	Pearson Correlation	1	.869
	Sig. (2-tailed)		.056
	N	63	5
IL5_Quant	Pearson Correlation	.869	1
	Sig. (2-tailed)	.056	
	N	5	10

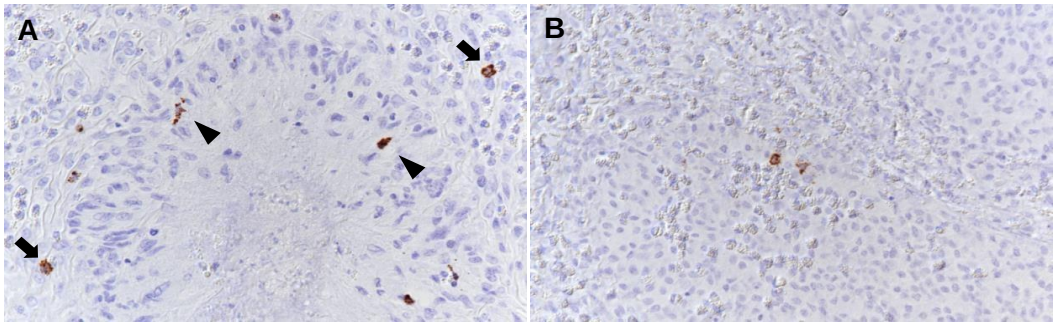


Statistical test (Pearson correlation).

3.6.6 Expression of interleukin 13 (IL-13)

EG showed intense IL-13 expression, mainly through lymphocytes and some palisading macrophages (Figure 63, A). In MCT, expression was less intense and restricted to some scattered lymphocytes (Figure 63, B).

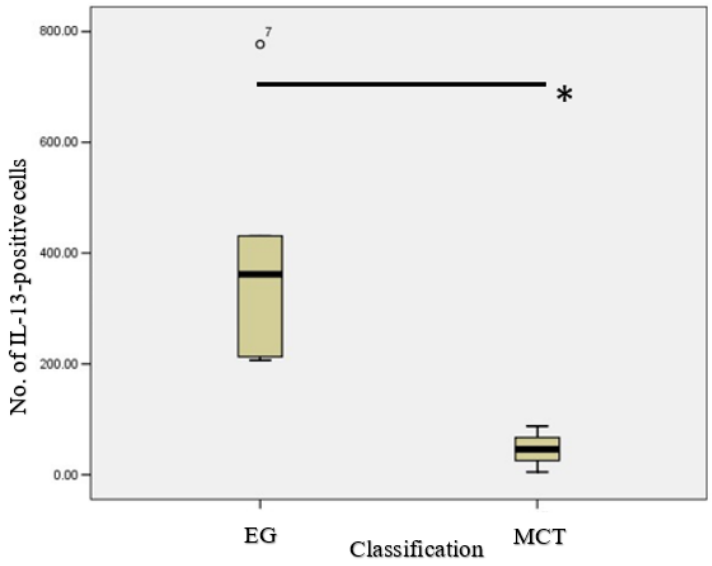
Figure 63: IL-13. (A) Case no. 09L-3297, category I. (B) Case No. 06L-1257, category IV



(A) Intense IL-13 expression, mainly by lymphocytes (arrows) and fewer macrophages and palisading histiocytes (arrowheads). (B) A low expression of IL-13 was observed in MCT, restricted to some scattered lymphocytes. mRNA-ISH, A400x, B 400x.

The difference in the number of IL-13 positive cells was statistically significant (Figure 64).

Figure 64: Box and whisker plots to illustrate the difference in IL-13 positive cells between EG (n=5) and MCT

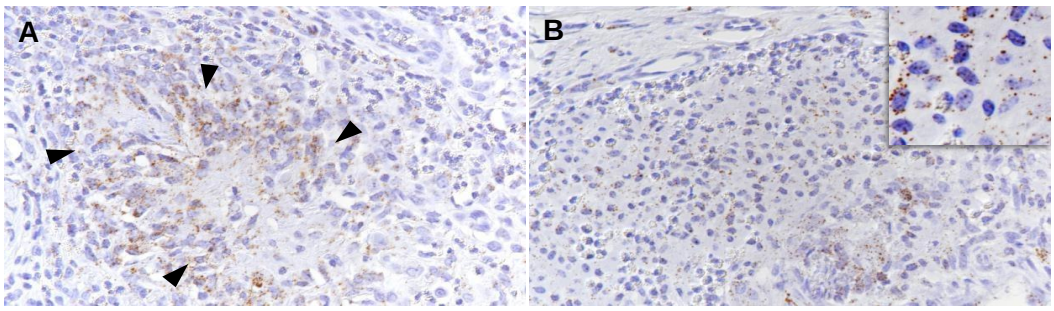


Statistical test (Mann-Whitney U) and the number representing outlier.

3.6.7 Expression of transforming growth factor beta (TGF-b)

TGF-β was found to be constitutively expressed in many different cell types, including mast cells and palisading macrophages in EG. Due to this generalised expression, a quantitative assessment was not attempted (Figure 65).

Figure 65: TGFb. Generalised expression of TGFb in many cell types, including palisading histiocytes in EG (arrowheads)

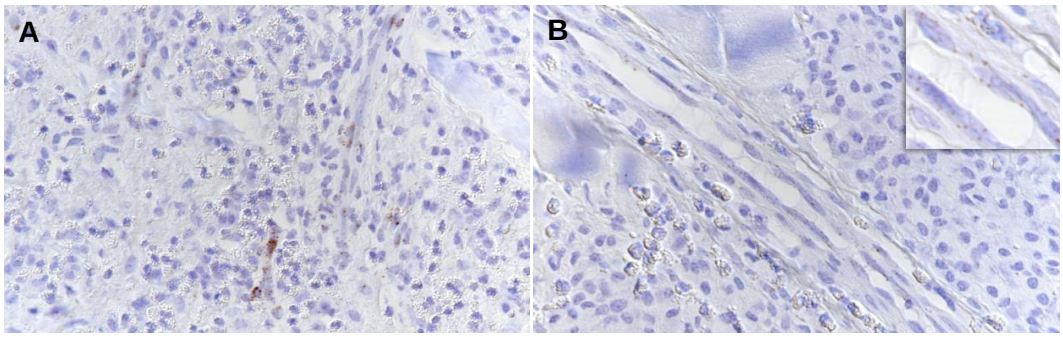


(A), Case No. 13L-4160, category I and mast cells (inset); (B), Case No. 11L-4696, category III. mRNA-ISH, A 400x, B 400x and 1000x (inset)

3.6.8 Expression of stem cell factor (SCF/cKIT-L)

SCF was found to be weakly constitutively expressed by endothelial cells and some vascular smooth muscle cells. Due to this generalised expression, a quantitative assessment was not attempted (Figure 66).

Figure 66: SCF. (A) Case No. 15L-4903, Category I. (B) Case No. 06L-1257, Category IV



Generalised weak expression of SCF in all cases in endothelial cells and some vascular smooth muscle cells. mRNA-ISH, A 400x, B 600x and 1000x (inset).

Table 19: Summary of the results obtained for the expression of cytokines and chemokines in EG and MCT

Marker / Cell type	MC	EO	MΦ	LC	EC	pHC		FB	OC	Predominant in
RANTES (CCL5)				✓		✓				EG?
Eotaxin (CCL11)			✓	✓				✓		Both (EG?)
MIP1α (CCL3)			✓	✓		(✓)				EG?
IL-4				✓						No role
IL-5	✓			✓						MCT
IL-13			(✓)	✓		(✓)				EG >> MCT
TGF-β	✓		✓	✓	✓	✓		✓	✓	Not clear
SCF/cKit-L					✓					Not clear

Abbreviations (cell type): Mast cells (**MC**), eosinophils (**EO**), macrophages (**MΦ**), lymphocytes (**LC**), endothelial cells and vascular smooth muscle cells (**EC**), palisading histiocytes (**pHC**), fibroblasts (spindle cells) (**FB**), other cell types (**OC**).

Abbreviations (markers): **RANTES** (*regulated on activation, normal T cell expressed and secreted*), **MIP1α** (*macrophage inflammatory protein-1α*), **SCF/cKIT-L** (*stem cell factor/c-KIT ligand*), **TGF-β** (*transforming growth factor-ß*).

3.6.9 Correlation between inflammatory cells and cytokine/chemokine expression

When the inflammatory cells and the different cytokines and chemokines were analysed alongside each other to test for any correlation (Table 20), it became obvious that T cells (CD3-positive) were associated with RANTES and IL4 expression (*green shadow*), and B cells (CD79-positive) were associated with RANTES, eotaxin, IL-4, and IL-13 expression (*blue shadow*). There was a direct correlation between the increase of eotaxin and IL-4 expression (*yellow shadow*), the increase of RANTES and IL-4 (*grey shadow*), and the increase in RANTES and IL-13 expression (*red shadow*).

Table 20: Cytokines and Inflammatory cell correlation (all cases) Spearman's Rho

Correlations

		CD3_ LowPower	CD79a_ LowPower	Iba1_ LowPower	Rantes_ Quant	Eotaxin_ Quant	Mip1a_Quant	IL4_Quant	IL5_Quant	IL13_Quant
CD3_LowPower	Pearson Correlation	1	.423**	.260*	.694*	.580	.622	.763*	-.332	.526
	Sig. (2-tailed)		.000	.012	.026	.079	.074	.028	.348	.118
	N	95	94	92	10	10	9	8	10	10
CD79a_LowPower	Pearson Correlation	.423**	1	.011	.683*	.654*	-.215	.737*	-.354	.654*
	Sig. (2-tailed)	.000		.921	.029	.040	.579	.037	.315	.040
	N	94	94	91	10	10	9	8	10	10
Iba1_LowPower	Pearson Correlation	.260*	.011	1	.644	.159	.361	.431	-.013	.645
	Sig. (2-tailed)	.012	.921		.061	.683	.380	.334	.974	.061
	N	92	91	92	9	9	8	7	9	9
Rantes_Quant	Pearson Correlation	.694*	.683*	.644	1	.292	.255	.765*	-.341	.875**
	Sig. (2-tailed)	.026	.029	.061		.413	.509	.027	.335	.001
	N	10	10	9	10	10	9	8	10	10
Eotaxin_Quant	Pearson Correlation	.580	.654*	.159	.292	1	.247	.739*	-.251	.114
	Sig. (2-tailed)	.079	.040	.683	.413		.522	.036	.483	.754
	N	10	10	9	10	10	9	8	10	10
Mip1a_Quant	Pearson Correlation	.622	-.215	.361	.255	.247	1	.407	-.182	-.019
	Sig. (2-tailed)	.074	.579	.380	.509	.522		.317	.640	.961
	N	9	9	8	9	9	9	8	9	9
IL4_Quant	Pearson Correlation	.763*	.737*	.431	.765*	.739*	.407	1	-.327	.503
	Sig. (2-tailed)	.028	.037	.334	.027	.036	.317		.430	.203
	N	8	8	7	8	8	8	8	8	8
IL5_Quant	Pearson Correlation	-.332	-.354	-.013	-.341	-.251	-.182	-.327	1	-.531
	Sig. (2-tailed)	.348	.315	.974	.335	.483	.640	.430		.114
	N	10	10	9	10	10	9	8	10	10
IL13_Quant	Pearson Correlation	.526	.654*	.645	.875**	.114	-.019	.503	-.531	1
	Sig. (2-tailed)	.118	.040	.061	.001	.754	.961	.203	.114	
	N	10	10	9	10	10	9	8	10	10

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

CHAPTER 4: DISCUSSION

4. Discussion

The following discussion is based on the results obtained after the re-examination of the cases, following the creation of the categories and groups noted in Chapter Three.

4.1 Clinical data

The clinical data collected in this study was based on the information provided by the referring veterinarian. Evaluation of the clinical data and case history information of all the 191 cases initially examined was undertaken without considering the final diagnosis after re-examination showed Thoroughbred (cross) and Arabs (cross), followed by Warmbloods, were most frequently represented and that males were more frequent in the study population than females. These results were confirmed when a subpopulation of 95 cases (for which a final diagnosis was obtained) were assessed.

4.1.1 Breed, age and sex in horses with eosinophilic granuloma

In the literature, eosinophilic granuloma had been reported as representing between 3.5% and 12% of submissions to diagnostic laboratories (Valentine, 2005, Schaffer *et al.,* 2013). Breed predilections had not been reported (Stannard, 2000; Pilsworth and Knottenbelt 2005; Scott and Miller 2011b, Wobeser 2015). In addition, investigations into nodular skin disease in horses did not identify any breed to be predominant in a cohort of 68 horses (van Der Zaag and Sloet Van Oldruitenborgh-Oosterbaan, 2012). In the present study, Thoroughbreds (cross) and Warmbloods, followed by Arabians (cross) were the most frequently represented breeds among horses with a final diagnosis of EG. However, the study also showed that a large variety of different breeds could be affected. Nonetheless, even without performing a statistical risk association, our results suggest that the above listed breeds were overrepresented. The reason for the apparent higher incidence of EG in these three breeds is unclear. However, their frequency in the study cohort might simply represent their frequency among the horse population in the UK. This suggestion is given greater validity by a recent study that investigated global horse populations

and tried to identify those breeds at risk of extinction. That study showed that the prevalence of different breeds varies around the world and not only showed that Arabians and Thoroughbreds are the most common breeds in European countries, but also that Warmblood horses that originated in Europe now represent 18% of horse breeds in the world (Khadka, 2010). Another reason could be that these represent more expensive horses for which owners would not spare the costs of a histological examination when a skin lesion was removed. In any case, a lack of a breed predilection for equine EG is in line with the findings in other species affected by eosinophilic granuloma: in neither cats nor dogs has there been any evidence of a breed predilection for EG (Bonello *et al.*, 2012, Buckley and Nuttall, 2012, Mauldin and Peters-Kennedy, 2016).

Most previous studies on equine EG did not find evidence of an age predilection (Stannard, 2000, Pilsworth and Knottenbelt, 2005, Scott and Miller, 2011b, Wobeser 2015), though one reported age as a possible factor to develop EG; the author found that horses affected with EG were predominantly younger than 10 years (Valentine, 2005). Another study found that the mean age of horses with EG was 8.3 years, with an age range of 3-14 years (Van Der Zaag and Sloet Van Oldruitenborgh-Oosterbaan, 2012). In our study cohort, the mean age of horses with EG was 9 years, which is similar to the previous study, but showed a wider age range (2- 23 years) (Van Der Zaag and Sloet Van Oldruitenborgh-Oosterbaan, 2012). There are differences compared to our study: first of all, we re-classified true EG and excluded lesions where aggregates of mast cells were present. Secondly there are a range of possible factors that may have influenced the results in the previous studies: Valentine (2005) confirmed that the number of EG was relatively small and that the data might be biased by the low numbers. Similarly, Van Der Zaag and Sloet Van Oldruitenborgh-Oosterbaan (2012) had a cohort of only seven cases examined within eight years from among a study population of 68 horses. Considering that EG is looked upon as a common disease in horses, it is surprising that these studies dealt with such low case numbers. However, it was suspected that attending veterinarians felt competent to make a diagnosis without a surgical biopsy when they observed grossly suggestive lesions in the saddle area or girth region (Van Der Zaag and Sloet Van Oldruitenborgh-Oosterbaan, 2012). The present study generated data from a substantially larger number of EG cases (n=98) and applied

a very strict diagnostic regime. It follows, *ceteris paribus*, that the data obtained is more likely to be more representative of the true age range of EG. However, it also confirms EG as a condition of middle-aged horses, though with a wide general age range. It has previously been argued that the incidence and nature of inflammatory skin lesions in horses may be influenced by geographical differences (Valentine, 2005), and that environment and exposure to different causative agents may modulate the incidence of EG irrespective of the age (Schaffer *et al.,* 2013). In cats, eosinophilic granuloma can occur at any age but is more common at younger ages (Bonello *et al.,* 2012, Mauldin and Peters-Kennedy, 2016). In dogs, most authors agree that there is no age predilection (Vercelli, Cornegliani *et al.,* 2005, Mauldin and Peters-Kennedy, 2016). That said, a few studies reported that EG is more common in young male Siberian Huskies and Cavalier King breeds (Bredal *et al.,* 1996).

Most previous studies on equine EG did not report any sex predilection (Stannard, 2000, Pilsworth, and Knottenbelt, 2005, Scott and Miller, 2011b, Schaffer *et al.,* 2013, Wobeser, 2015), though one found evidence that EG is more common in males (Valentine, 2005). Similarly, the current study does not suggest any sex prevalence. This is similar to the previous studies upon dogs and cats, where one study reported a higher incidence in females (Bonello *et al.,* 2012), followed by another study which concluded that there was no sex predisposition in cats and dogs with EG (Mauldin and Peters-Kennedy, 2016).

4.1.2 Breed, age and sex in horses with cutaneous mast cell tumour

In contrast to EG, mast cell tumousr are relatively rare, representing only 3-7% of cutaneous and muco-cutaneous neoplasms (Scott and Miller, 2011c, Kiupel, 2017). In addition, in our study, MCT are far less frequent than EG (36% vs 64%).

Most studies of equine MCT did not observe a breed predilection (Scott and Miller 2011c, Van Der Zaag and Sloet Van Oldruitenborgh-Oosterbaan, 2012, Schaffer, 2013). However, some recent studies have suggested that Arabians were more frequently affected. In one study this association was not statistically significant (Mair and Krudewig, 2008), whereas another showed that Arabian horses were 5.1 times more likely to develop a MCT than other equine breeds (Clarke *et al.,* 2014). A third study found MCT most frequently in Arabian (cross) and Thoroughbred

(cross) horses, but without a statistical risk association (Ressel *et al.,* 2015). The latter results were carried further when a statistical correlation between breed and MCT was reported for Thoroughbred (cross) and Arabian (cross) horses (Knowles *et al.,* 2016). This study also showed associations between MCT and Cob (cross) horses (Knowles *et al.,* 2016).

Our results are in line with those of the recent studies as Thoroughbreds and Arabian horses were the most affected breeds with MCT, followed in frequency by Welsh horses. The reason for the obvious MCT predisposition of different breeds might be due to a number of factors including the geographical area in which the case cohort was collected, and the length of the study. Interestingly, one study reported no cases of MCT in Thoroughbreds, although this breed accounted for 50% of the population (Scott and Miller, 2011c). As an additional observation, it is interesting to note that similar breeds are overrepresented in both EG and MCT. This supports the contention that one reason for overrepresentation might be a common genetic background.

In the present study population, horses with MCT exhibited a higher mean age (11 years) compared to those diagnosed with EG (9 years). This is in line with previous studies that have shown increasing age in horses to be a risk factor in the develop ment of neoplasia in general (Silva and Furr, 2013, Knowles). In addition, several studies on MCT showed mean ages ranging between 7 and 15 years, with age ranges from 1 to 30 years (Valentine, 2005, Clarke *et al.,* 2014, McEntee, 1991, Van Der Zaag and Sloet Van OldruitenborghOosterbaan, 2012, Millward et al, 2010). In our study, we observed a similarly wide age range (3 – 25 years) which suggests that MCT may develop at any age in horses, like EG.

For MCT, the present study found male horses to be more frequently affected than females. This finding is in agreement with previous studies (Mair and Krudewig 2008, Scott and Miller, 2011c, Wobeser, 2015, Clarke *et al.,* 2014, Ressel, 2015). However, one study disagreed and found no sex predisposition in a 10 year retrospective cases series; MCT comprised 2% of all tumours in the data set (Schaffer, 2013). No gender difference in dogs and cats affected with MCT has been reported (Blackwood, 2015, Kiupel, 2017). So far, there have been no studies into the possible reasons for this difference. The present study does not address this

feature. However, considering what is known from humans regarding the role of mast cells in hypersensitivity and the crosstalk between eosinophils and mast cells, several studies have found correlations between sex and allergic inflammation, such as asthma involving oestrogen, eosinophils and mast cells (Cai *et al.*,2012, Keselman and Heller, 2015). A study demonstrated the possible role that oestrogen in the stimulation of Th2 cytokine production and eosinophil regulation in developing chronic inflammation, such as allergy and asthma, changes reported to be more frequent in females (Cai *et al.,* 2012) but there are no data in regard to tumour development. However, oestrogen affects eosinophils in different ways and, depending on the receptors engaged, this can be different in different animal, and also differ between tissues in which the signalling is taking place, it also depends on whether the cells are analysed in vivo or in vitro (Keselman and Heller 2015). It is possible that such different signalling plays a role in the early stages of MCT development, but this needs further study.

4.1.3 Body location, size and number of lesions

Eosinophilic granuloma has been reported to be most commonly diagnosed on the neck and trunk (withers and back) of horses (Stannard, 2000, Pilsworth and Knottenbelt, 2005, Scott and Miller, 2011b, Mauldin and Peters-Kennedy, 2016). However, they can occur anywhere and may also be multifocal (Stannard, 2000, Mauldin and Peters-Kennedy, 2016). One study reported the specific location of seven EG cases and found four in the axillary region, and one each on nose, shoulder and buttock (Van Der Zaag and Sloet Van OldruitenborghOosterbaan, 2012). In the present study, the trunk was the most common location, but eyelid, head, and limb and in conjunctiva were also affected in some cases.

Equine cutaneous MCT are commonly found on the head, trunk and limbs (Mair and Krudewig, 2008, Scott and Miller, 2011c, Wobeser, 2015, Kiupel, 2017), but have also been reported in the eye, affecting the conjunctiva, sclera, *Membrana nictitans*, limbal area, cornea, third eyelid and the entire globe (Martin and Leipold 1972, Hum and Bowers, 1989, McEntee, 1991, Ward *et al.,* 1993, Malikides *et al.*, 1996, Bosch and Klein, 2005, Mair and Krudewig, 2008, Smiech *et al.,* 2009, Halse *et al.,* 2014, Kiupel, 2017, Shnaiderman-Torban *et al.,* 2017, Flores *et al.,* 2017). Tumours typically present as solitary immovable nodules on the head, trunk, neck,

and limbs, where they are often found close to joints (Scott and Miller, 2011c, Kiupel, 2017). Most tumours are neither painful nor pruritic and may be hyperpigmented, alopecic, or ulcerated (Mair and Krudewig, 2008, Kiupel, 2017).

Usually, mast cell tumours in horses are benign and excision is curative as recurrence is uncommon (McEntee, 1991, Kiupel, 2017). Apart from affecting the skin, MCT occur at other body sites in horses, such as the mucous membranes and even testes (Brown *et al.*,2008), bone (Ritmeester *et al.*,1997), synovial membranes (Taylor *et al.*, 2005) and in multicentric forms (Reppas and Canfield 1996, Tan et al.2007, Millward *et al.*,2010). Interestingly, one study even reported there was no apparent site of predilection for mast cell tumours (Schaffer *et al.*, 2013). In the species most commonly affected by MCT, dogs and cats, MCT develop most frequently in the skin and subcutis at any site of the body (Blackwood et al, 2012, Kiupel, 2017, Sabattini and Bettini, 2010, Blackwood, 2015).

The results of the present study also found the head to be the most frequently affected site, which is in line with previous studies (Valentine, 2006, Clark et al, 2014, Ressel *et al.*, 2015). However, different from others reports (Mair and Krudewig, 2008, Hales *et al.*, 2014, Knottenbelt *et al.*, 2015), the eye (conjunctiva and eyelid) was the second most commonly affected location (18.2%), followed by trunk, neck, and limbs. In the eye, the limbal area and occasionally the eyelids were found to be affected by MCT (Knottenbelt *et al.*, 2015). There are a few case reports describing MCT in the conjunctiva (Hum and Bowers, 1989, McEntee, 1991) and eyelids (Van Der Zaag and Sloet Van Oldruitenborgh-Oosterbaan, 2012, Smiech *et al.*, 2009, Shaiderman-Torban *et al.*, 2017). MCT located in the eye belonged to Categories II-V in our population. The reason why the eye was more affected in our results compared to other studies is unclear. Interestingly, all but one ocular MCT case in existent literature were reported in females horses when the sex was stated (Worad *et al.*, 1993, Hum and Bowers, 1989, Smiech *et al.*, 2009; Halse *et al.*, 2014, Shaiderman-Torban *et al.*, 2017, Flores *et al.*, 2017). In contrast, there is evidence of a predilection for male horses (Mair and Krudewig, 2008, Scott and Miller, 2011c, Clark et al, 2014, Kiupel, 2017). In our cohort of MCT in the eye, four were found in females (conjunctiva (3) and eyelid) and six in males (conjunctiva (2) and eyelid (4), which does not confirm the trend indicated in the literature, suggesting

that MCT in the eye do not differ from those in other locations. The same is true with regard to age; the mean age of the ocular cohort was 11.5 years, and the range was 10 – 15 years.

In the present study, the size of EG ranged from 5mm to 9cm in their major diameter, which is similarly to the size described (less than 1cm to more than 10cm) reported by, amongst others, Scott and Miller (2011b), Wobeser, (2015), and Mauldin and Peters-Kennedy, (2016). Similarly, MCT were reported to range from 0.5mm to 20cm (Mair and Krudewig, 2008, Scott and Miller, 2011c, Clark *et al.*, 2014, Ressel *et al.*, 2015). In the present study the MCT were equally variable, with size ranging from 2mm to 10cm.

In the literature, there is no data as to the prevalence of single or multiple lesions with regards to EG and MCT. However, it is known that EG can present as single and multiple lesions (Scott and Miller, 2011b, Mauldin and Peters-Kennedy, 2016), while MCT usually occur as solitary lesions (Mair and Krudewig, 2008, Scott and Miller, 2011c). In the current study, EG were more common as single lesions, but also occurred as two or more lesions (25 cases). Interestingly, male horses were particularly overrepresented in cases of multiple lesions (60% of the males). The vast majority of MCT were single lesions (86%), with only 7 cases of multiple lesions.

4.2 Morphological features

The systematic morphological re-examination of a uniquely large cohort of 191 cases with an initial histopathological diagnosis of MCT and/or EG revealed a number of features that would suggest strict criteria for a diagnosis of EG and MCT, respectively. Ultimately, the presence of mast cell aggregates (MCA) would lead to the diagnosis of MCT which could comprise more or less large proportions of EG components, whereas the diagnosis EG would exclude the presence of MCA. Based on the subsequent detailed examination of 95 cases, five categories of lesions were defined which were then grouped together into three groups of lesions and, ultimately, allocated to the two diagnoses, EG and MCT.

Using this new scheme, the original diagnosis in the diagnostic reports was changed in 11 lesions (10.6%) from EG to MCT lesions, and from MCT to EG in 1% of

lesions. In most instances, this implied a shift from Category I to II and was therefore based on the identification of MCA within large EG areas, leading to reclassification from EG to MCT. The reasons for the reclassification could be the absence of strict criteria for the diagnosis so far, and an overlooking of MCA in EG. In the section that follows, the morphological features leading to the new classification are discussed.

4.2.1. Size of lesion and proportion of EGC and MCA

The results of the present study indicate that there are differences in lesion size and the number of EGC and MCA between categories. Lesions in Category I (i.e. "pure" EG) were predominantly small, and there was an increase in size from MCT belonging to Categories II, III, and IV. This suggests a potential temporal relation, making Categories II, III, and IV consecutive stages of MCT proliferation. Interestingly, Category V lesions were again smaller. This could be due to two reasons: first, that Category V lesions ("pure" MCT) could represent an earlier stage of Category II and III lesions. Second, that MCT size is influenced mainly by the associated EGC (present in Categories III and IV, but not in V). However, when the results of the measurements of EGC and MCA in the same lesion were compared, it appears that the component that drives the size of the lesion in Categories II and III is indeed the MCA. Further investigations with a known timeframe of lesion development is needed to better understand these differences.

4.2.2. Location of lesions in skin and subcutis

The morphological evaluation showed that the deep dermis is the most common location of all five categories, followed by the mid dermis and the subcutis. Despite this, it was also evident that lesions were rarely confined to one layer. As a consequence, when the "depth score" was applied – it considered the depth of the lesion weighing all the layers involved, there was no difference between categories, groups or EG vs. MCT. This suggests that lesions can, in principle, extend to all the different layers. However, there is a clear tendency of lesions in Category I ("pure" EG) to extend to the superficial dermis, which was not seen in the lesions found in the other categories. This adds more precise information to that which is known from existent literature and states that EG exhibit nodular to diffuse areas of eosinophilic, granulomatous inflammation in the dermis and often the panniculus

(Scott and Miller, 2011b, Wobeser, 2015, Mauldin and Peters-Kennedy, 2016). It is also in line with another finding in the present study, the involvement of hair follicle in EG (see Chapter 4.2.4.).

MCT are described as dermal and subcutaneous mast cell nodules (Doran and Collins, 1986, McEntee 1991). The subcutaneous layers are often involved, and the underlying superficial musculature may sometimes be infiltrated as well (McEntee 1991, Ward *et al.*,1993, Mair and Krudewig, 2008, Millward *et al.*, 2010, Scott and Miller, 2011c). Our study confirms the previous findings. According to Mauldin and Peters-Kennedy, (2016) mast cells are present in large numbers in dermis and subcutis of horses (Mauldin and Peters-Kennedy, 2016). This finding suggests these cells are likely to be the cells of origin of these neoplasms. Furthermore, Hales (2014) and Flores (2017) suggested that normal mast cells are present in the lamina propria of the conjunctiva, and these are likely to be the origin of this tumour in equine eyes (Hales *et al.*, 2014, Flores, 2017).

4.2.3. Presences of individual mast cells in areas of EG

The present study found individual mast cells scattered throughout only 13 lesions (42%) of Category I (EG), while they were present in all lesions within Category II (MCT with EG > MCT components), and absent in all the other categories.

Infiltrating mast cells have previously been described in EG (Brown, *et al.*, 2007, Johnson, 1998). However, these studies did not apply the strict criteria that the present study has adopted. This, in turn, precludes a direct comparison. Our finding of individual mast cells within pure and thereby MCA-free EG is surprising as mast cells are not likely to play any role in the pathogenesis of eosinophilic granuloma formation. In this context it is possible that mast cells themselves or TAMs play a role in developing EG in MCT (see further discussion in Chapter 4.3.3.).

Among MCT, only Category II lesions with small mast cell aggregates as the only MCT components exhibited individual mast cells within the EGC areas. If it is hypothesised that Category II lesions represent earlier MCT stages than Category III and IV lesions, then it would follow that the the individual mast cells are single cells scattered part of the MCA in the early stages of lesions. However, such a hypothesis does not fulfil the general dogma of neoplastic cell growth where a

clonal and, therefore, centrifugal expansion is believed to be the driving force of the nodular formation of lesion. However, it is reported that in MCT neoplastic mast cells are arranged not jujst in ribbons or nests but may also sometimes be more individual (Clark *et al.*, 2014, Kiupel, 2017). These could represent individual neoplastic cells or they might have invaded adjacent tissue.

4.2.4. Association of EG with the hair follicle

The literature does not report on an association between EG and hair follicle structures, a feature for which the results of the present study provide strong evidence. The present study observed a direct spatial association identified in more than half (52%) of the Category I lesions (EG) with hair follicle structures. UIt also recorded that this was entirely absent in all other Categories (II-IV, MCT). This result suggests that horses may have a specific form of furunculosis that is driven by eosinophils and macrophages. Considering the two main cellular components of EG, two potential pathogenetic mechanisms can be considered to underpin at least a proportion of EG cases. This could represent a different first line of sight against bacteria, which could represent the initiating trigger for the formation of the EG in Category I lesions.

Interestingly, in some EG, free hair shafts have been reported in the dermis. These are usually linked to a history of body clipping prior to the occurrence of the lesions, which suggests a causal relationship (Stannard, 2000, Scott and Miller, 2011b) and that traumatically introduced hair fragments can provoke the inflammatory response (Mauldin and Peters-Kennedy, 2016).

Folliculitis, i.e. inflammation of the hair follicle, is further classified into mural folliculitis (inflammation of the follicular epithelium), luminal folliculitis (inflammation in the follicular lumen), and perifolliculitis (inflammation around, but not significantly impinging on the follicle). Furunculosis is often associated with dermal inflammation which follows from the destruction of the follicular epithelium and leads to the release of luminal content into the dermis. Furunculosis has been observed in bacterial infections, dermatophytosis, demodicosis, and trauma (Scott and Miller, 2011a, Mauldin, and Peters-Kennedy, 2016). Furunculosis can also occur as a foreign body-type reaction to free keratin and hair shafts and is often associated with moderate numbers of eosinophils (Scott and

Miller, 2011a). In this context it is noteworthy that eosinophils play a relevant role in host defence and diseases: studies in humans have demonstrated that eosinophils contribute to defence against multiple (exogenous) agents, such as parasites, bacteria, viruses and fungi (Park and Buchner, 2010, Shamri *et al.,* 2011). Eosinophils have antibacterial capacities, through ingestion of bacteria, or the release of bactericidal mediators (Shamri *et al.,* 2011).

Eosinophil granules play a key role against bacteria. Both major basic protein (MBP) and eosinophil cationic protein (ECP) were shown to cause permeabilisation of the outer and inner membranes of *Escherichia coli*. The same study also found evidence that the granules can translocate ECP and MBP into phagocytic vacuoles, thereby supporting ingestion of phagocytosed bacteria (Lehrer *et al.,* 1989). On the other hand, Nakajima *et al.,* (2001) showed that eosinophil RNases have in vitro bactericidal activity against *E.coli* and *Staphylococcus aureus*. In fact, through the production of superoxide in a nicotinamide adenine dinucleotide phosphate (NADPH) oxidase- and eosinophil peroxidase (EPO) - (but not nitric oxide) dependent manner, eosinophils can rapidly kill *E. coli* in *in vitro* studies (Persson *et al.,* 2001). In addition, *in vitro*, all cationic granule proteins of eosinophils have shown to have bactericidal activity (Shamri *et al.,* 2011).

Furthermore, both Gram-negative and Gram-positive bacteria were shown to activate eosinophils, particularly *E. coli*, which can induce eosinophil chemotaxis, degranulation and respiratory burst (Svensson and Wenneras 2005, Hogan, *et al.,* 2013). MBP and EPO can be released from eosinophils even in the presence of low bacterial quantities, whereas higher bacterial numbers are required for the release of ECP (Svensson and Wenneras, 2005).

Similarly, isolated mouse eosinophils were shown to possess anti-pseudomonal *in vitro* properties through the release of cationic secondary granule proteins (Linch *et al.*,2009). In addition, hypereosinophilic IL-5 transgenic mice exhibited a better ability to clear *Pseudomonas aeruginos*a from the peritoneal cavity, while eosinophil-deficient mice showed a weak ability to clear bacteria even after the adoptive transfer of eosinophils. The administration of material from eosinophil granules increased the ability of bacterial clearance *in vivo* (Linch *et al.,* 2009). Eosinophils are also described to behave like neutrophils in forming NETs

(Neutrophilic extracellular traps) – like structured (i.e. mitochondrial DNA and granule proteins) that can trap and kill bacteria (Yousefi *et al.*, 2008).

Esinophils also express the necessary cellular machinery (innate immune receptors, proinflammatory cytokines, antibacterial proteins and DNA traps) to mount an efficient antibacterial response. However, the rapid decline in eosinophil numbers following acute systemic bacterial infection suggests a very limited role for eosinophils in bacterial responses, leaving many open questions on the role of eosinophils in bacterial infection also in humans (Hogan, *et al.*, 2013).

This large body of literature suggests that there is major role for eosinophils in antimicrobial defence. From this it can be concluded that eosinophils might contribute to effective immunity against bacteria through the coordination of antibacterial inflammatory cascades as well as the recruitment and activation of other cells (i.e. macrophages), via cytokines, chemokines and lipid mediators. It is possible that in horses, eosinophils are more involved in the antimicrobial defence than in other species. This, it is suggested, is particularly true with regard to contact infections of the hair follicles which can lead to an EG when the bacteria cannot be fully eliminated and the process becomes chronic.

4.2.5. Focal mineralisation

Focal mineralisation was a frequent feature of Category I lesions (64%). It was found in only 8%-21% of Category II-IV lesions and was entirely absent in Category V ("pure" MCT). The statistical evaluation confirmed a significant association of mineralisation with EG, which was not seen in MCT (Groups 2 and 3). Mineralisation was only seen in areas of EGC, and may possibly be interpreted as dystrophic mineralisation.

In general, mineralisation (calcium deposits) is a term used to describe the deposition of insoluble, inorganic minerals which consist of calcium in combination with phosphate or carbonate. The terms 'mineralisation' and 'calcification' are often used interchangeably as calcium is the dominant mineral in the deposit. Mineralisation is further classified into four basic forms; dystrophic, metastatic, idiopathic, and iatrogenic (Reiter *et al.*, 2011, Kumar *et al.*, 2015, Hargis and Myers, 2017).

The literature describes marked dystrophic mineralisation in older EG lesions and comments that this may be common and can lead to misdiagnosis as calcinosis circumscripta or mast cell tumours (Scott and Miller, 2011b, Wobeser, 2015, Mauldin and Peters-Kennedy, 2016). In equine MCT, dystrophic mineralisation is described to be frequently seen in areas of collagen degeneration and necrosis (McEntee 1991, Ward *et al.*,1993), old lesions seem to predominantly consist of fibrosis and dystrophic mineralisation, with only small packages of mast cells remaining (Mair and Krudewig, 2008, Scott and Miller, 2011c, Mauldin and Peters-Kennedy, 2016). Some tumours may be dominated by an extensive stromal response (i.e. collagen bundles, fibroplasia), areas of dystrophic mineralisation, and large aggregates of eosinophils and foci of necrosis (Kiupel, 2017).

So far, no studies have found differences in the presence and extent of mineralisation between EG and MCT.

In the previous studies the HE stain was used as a means of identify mineralisation. The mineralised material itself varies from amorphous to granular and is variably basophilic (depending on the stage of mineralisation). Authors report that in granular areas, one can see a transition from intact eosinophils, to eosinophil granules and nuclear debris, to completely amorphous material (Scott and Miller, 2011a). In dystrophic mineralisation, calcium salts deposit as basophilic, amorphous, granular material along collagen fibrils (Mauldin and Peters-Kennedy, 2016, Scott and Miller, 2011a). However, the HE stain does not allow us to specifically identify calcium salt deposition. As a consequence, areas of mineralisation may not be evident or may be misinterpreted, and special stains such as the von Kossa stain should be applied (Zachary and McGavin, 2012).

The present study extended its investigation into the focal mineralisation in EG and MCT. We hypothesised that if there is a link between age of the lesion and the extent of mineralisation (as suggested in previous publications), there should also be a link between the size of the lesion and the extent of mineralisation. However, the statistical analysis found no association between the presence of mineralisation (von Kossa positive areas) and the size of the EGC. Further analysis based on the purple stained areas in Lendrum's' stained sections (see Chapter 4.2.12.) suggested

that there is a link between size of EGC and mineralisation only in Category I lesions, but not in the lesions of the other categories.

All these results suggest that mineralisation is a process that is relevant only in Category I lesions (EG). This is because it is only in this category that it is directly proportional to the size. This further implies that it is increasing with the age of the lesion. In contrast, in lesions of Category II-IV, mineralisation is not always part of the process of eosinophil degeneration within the granuloma core formation, while in Category I the vast majority of lesions exhibit mineralisation. Despite the presence of cellular debris within the granuloma core, (which could suggest that the mineralisation is dystrophic and related to cell necrosis), a different pathogenetic mechanism cannot be ruled out. This is particualrl true when one considers that primary degeneration of collagen can be a cause for the development of the eosinophilc granuloma in the first place (Stannard, 2000).

One of the hypotheses for the causes of EG is degeneration of collagen (Stannard, 2000). Mineralisation can affect individual or groups of collagen fibres, resulting in increased basophilia and fragmentation of fibres in HE-stained sections. Calcium deposits can trigger a granulomatous inflammatory response - as a foreign-body reaction to the deposit (Hargis and Myers, 2017).

Dystrophic mineralisation occurs because of injury or degeneration of cellular and extracellular components, with normal serum calcium concentrations and calcium metabolism (Kumar *et al.,* 2015, Hargis and Myers, 2017). It is often seen in granulomas and calcinosis cutis as well as with hyperadrenocorticism. Trauma can cause tissue mineralisation as a final consequence (Zachary and McGavin, 2012, Hargis and Myers, 2017). Metastatic mineralisation occurs with hypercalcemia due to abnormal concentrations of calcium metabolism, phosphorus, or vitamin D, without preceding tissue injury or degeneration. This type of mineralisation is usually seen in soft tissue with chronic renal disease or other pathology that leads to parathyroid hyperplasia (Zachary and McGavin, 2012, Hargis and Myers, 2017). The term “idiopathic calcification” is used when there is deposition of calcium salts without underlying tissue damage or abnormal calcium or phosphorus metabolism, with unknown causes. Iatrogenic calcification occurs in human patients that are

receiving calcium or phosphate containing substances (Zachary and McGavin, 2012).

It is clear that different factors can cause mineralisation in EG and MCT. Considering that IL-5 was observed expressed more frequently in MCT compared to EG (see Chapter 4.2.14.), and that mineralisation is associated with EG/EGC rather than with MCA, the following interpretation can be made: In view of the role of IL-5 in maintaining the viability of eosinophils, dystrophic mechanisms are likely to be the key to mineralisation in Category I lesions. However, further studies are needed in order to investigate whether or not this is correct and to ascertain as to whether different or additional mechanisms, such as collagen degeneration followed by mineralisation, are involved.

4.2.6. Focal ulceration

The present study identified focal ulceration of the skin in association with both EG and MCT. With EG lesions, skin and haircoat are typically unaltered, and the lesions are not associated with signs of pain or pruritus (Stannard, 2000, Scott and Miller, 2011b, Mauldin and Peters-Kennedy, 2016). However, if challenged by traumatic injury (which is a likely event due to the fact that they are exophytic), lesions may ulcerate and necrosis may develop as well as crusts and alopecia at the site of the nodules (Stannard, 2000).

In MCT lesions, the overlaying skin is usually normal and intact, but hyperpigmentation, alopecia or ulceration can occur (Riley *et al.*, 1991, Mair and Krudewig, 2008, Millward *et al.*, 2010, Scott and Miller, 2011c, Mauldin, and Peters-Kennedy, 2016, Kiupel, 2017). Lesions are generally painless or non-pruritic, but are reported to occasionally discharge caseous material (Mair and Krudewig, 2008, Scott and Miller, 2011c).

Based on the previous reports and our findings, ulceration is considered a secondary event that is most likely unrelated to the pathogenesis of the two conditions. Rather, it is perceived to be a consequence of mechanical forces (trauma) on the surface of the nodule; a likely event in horse skin.

4.2.7. Presence of collagen fibres within EGC areas

Collagen fibres or their fragments were found embedded in almost all EG lesions (93%) using Masson Trichrome-stained sections. In addition, Category I (EG only) showed significantly more collagen fragments within the granuloma core than the other categories within their EGC. This suggests that collagen may be involved in granuloma formation in true EG, but not in the development of EGC areas in MCT. However, it is possible that collagen is also an innocent bystander in some lesions, especially if not degenerated.

A link between degenerate collagen fibres and a granulomatous host response has been made before regarding collagen acting like a foreign body (Stannard, 2000). When previously reported, collagen fibres within equine EG appeared to be normal in size, surface contour, and consistency when stained with trichrome (Fernandez *et al.,* 2000, Scott and Miller, 2011a).

As a result, collagens fibres which have been shown to exhibit irregularities, granularity and fuzziness in HE-stained sections are likely to be coated with red-staining material, masking the classical green stain in Masson's trichrome stained sections. This phenomenon is, however, believed to not be necessarily associated with "collagen degeneration" or "collagenolysis" (Fernandez *et al.,* 2000, Scott and Miller, 2011a).

The term "flame figure" refers to an area of collagen which is surrounded by eosinophils and eosinophil granules, (Fondati *et al.,* 2001, Scott and Miller, 2011a, Scott and Miller, 2011b, Ackerman 2017). The eosinophilic material varies in shape (i.e. annular to oval, to a radiating configuration ("starburst") appearance). These areas consist of intact eosinophils, eosinophil granules and nuclear debris and completely amorphous material (Scott and Miller, 2011a, Ackerman, 2017). Collagen fibres within flame figures can appear normal, but can also be swollen, irregular or fuzzy, and granular (Fondati *et al.,* 2001, Scott and Miller, 2011a, Ackerman 2017).

Other findings regarding the structure of the collagen fibres are at ultrastructural level (using transmission electron microscopy) and were generated on feline eosinophilic granuloma (FEG) complex lesions. These indicate that fibres can be

disrupted, but not degenerated (Bardagi *et al.*, 2003). Furthermore, studies on Wells' syndrome in humans and FEG suggested that insoluble eosinophil granules provoke the granulomatous reaction seen around flame figures (Brehmer-Andersson *et al.*, 1986, Fondati *et al.*, 2001). In addition, recruitment and degranulation of eosinophils were suggested as the primary events in FEG and collagen fibres are unlikely to play any actual role in the pathogenesis of this disease complex. Rather, they act as bystanders that play a secondary role (Bardagi *et al.*, 2003).

The results of the present study indicate that collagen fibres are morphologically normal within the area of flame figures in the granuloma core in equine EG, which suggests no causative role of collagen in the granulomatous inflammation. It indicates, similar to what has been speculated for FEC, that eosinophil degranulation is indeed the primary event in the pathogenesis of EG. However, further studies are needed to elucidate this aspect.

4.2.8. Lymphocytes aggregates

Lymphocytes aggregates (which were characterised as consisting of B cells, (see Chapter 4.2.13.2 B cells) were mainly a feature of EG (Category I) rather than of MCT (Groups 2 and 3). This suggests that B cells' response dose not play a role in MCT.

Previous studies did not investigate the lymphocyte populations in equine EG and only reported that EG can contain lymphoid aggregates and that they may be prominent (Scott and Miller, 2011b, Mauldin and Peters-Kennedy, 2016). The present study confirms the presence of lymphoid aggregates in Category I (65% of lesions). These were almost entirely composed of B cells (see Fig. 3.42). In addition, a few lesions (three lesions in Category IV) exhibited lymphoid aggregates, a feature that has so far only been reported in feline MCT. A study in cats detected lymphocytic foci in 40% of well-differentiated MCT, 85% of pleomorphic MCT and in all atypical MCT. However, the potential role of these lymphoid aggregates in feline MCT is still unclear (Sabattini and Bettini, 2010). Due to the rarity of the phenomenon in our population, it cannot be excluded that this is an incidental and unrelated finding, especially since no statistical association

was found between lymphocytes aggregates and in any of the categories apart from Category I.

B cells play a defensive role against exogenous agents, such as bacteria, and activate the production of antibodies (i.e. through plasma cells) thereby supporting the immune function of T helper cells (Tizard, 2013, Murphy and Weaver, 2017). Considering the frequent association of Category I lesions (EG) with thesuperficial dermis and hair follicles with "furunculosis-like" granuloma formation discussed above, the presence of B cell aggregates further supports an exogenous cause for a proportion of EG cases.

4.2.9. Infiltrating viable eosinophils in MCA

The present study found evidence of an increase in the proportion of viable eosinophils infiltrating MCA within higher category numbers. There was aksi an increasingly large MCA component in the MCT. Within existent literature MCT are usually reported to contain large numbers of viable eosinophils between the neoplastic mast cells (Scott and Miller, 2011c). In both large and small animals, MCT are infiltrated by eosinophils, which is believed to be the effect of chemotactic factors and cytokines (Hill *et al.,* 1991, Riley *et al.*, 1991, Ritmeester *et al.*, 1997, Govier, 2003). In our study, the more MCA dominate the lesions compared to EGC, the more eosinophils are infiltrating the MCA, which suggests that mast cells play a relevant role in recruiting eosinophils into the lesion. As a consequence, in EG (Category I lesions), the recruitment of eosinophils into the lesion to form the core of the EG must be mediated by other cells and mechanisms. Considering the increased expression of IL-5 by tumour cells or TAMs in the MCT compared to EG, it is likely that cytokine plays a role in eosinophil recruitment into MCT, but not in EG. Indeed, IL-5 is the most important cytokine produced by mast cells in humans and animals and is particularly responsible for the survival of eosinophils (Tai *et al.,* 1991, Bagley *et al.,* 1997, Park and Bochner, 2010, IImarinen, *et al.*, 2014, Shen and Malter, 2015, Wen and Rothenberg, 2016).

4.2.10. Mitotic figures in MCA

The assessment of mast cells in MCT (Categories II-V lesions) for their proliferative activity based on the presence of mitotic figures showed obvious

differences between the categories, with significantly higher numbers in Category V lesions (8-9/10 HPF) compared to the number of lesions in all other categories, and in Category IV (4/10 HPF) compared to Category II (1-2/10 HPF). This result indicates that proliferative activity is proportional to the relative abundance of MCA in MCT.

Within existent literature only a few studies have investigated the mitotic activity in larger cohorts of MCT in horses. McEntee (1991) investigated 30 MCT and found that mitotic figures varied (1-10/HPF). Another study investigated 72 MCT and only found three (4%) with more than two mitoses in 10 HPF (Clark *et al.*, 2014). Similarly, Ressel *et al.,* (2015) investigated 45 cases and found 18 horses exhibiting a number of mitotic figures up to 6 per 10 HPFs. These findings suggest a variable growth rate in equine MCT. Further systematic studies are needed to assess whether this also results in differences in biological behaviour.

4.2.11. Macrophages phagocytosing eosinophils

In some MCT, macrophages engulfing eosinophils were observed. This was not a feature in EG. Several studies suggest that the phacogytosis of eosinophils follows programmed cell death (apoptosis) of these cells (Stern *et al.,* 1992).

Apoptosis is an essential mechanism to limit cell numbers in human and animals and is the most common form of physiological cell death (Park and Bochner, 2010). It has been proposed as a potential mechanism that contributes to the resolution of eosinophilic inflammation (Simon *et al.,* 1997). There is abundant data in humans and animal models which demonstrates the underlying mechanisms for the different pathways that lead to eosinophil apoptosis and death (Park and Bochner, 2010). Eosinophils die by passive apoptosis (spontaneous) which includes IL-5 and its signalling pathway, mitogen-activated protein (MAP) kinase, caspases, and Bcl-2 family members (Ilmarinen *et al.,* 2014, Park and Bochner, 2010). In contrast, active apoptosis involves Fas, Siglec-8 and Siglec-f, TGF-β, CD30, corticosteroids, lidocaine, and IRp60/CD300a (Park and Bochner, 2010).

The key cytokines for eosinophil survival are IL-5, IL-3 and GM-CSF with CD125, CD123 and CD116 as their respective receptors. These receptors have a specific α-chain, but share the β-chain which causes an overlap in their functions and leads to

mediate transduction signals to initiate apoptosis of eosinophils (Geijsen *et al.*, 2001, Shen and Malter, 2015). The intracellular pathways (Lyn, Jak2, Raf1 and MAP kinases) are vital to inhibit apoptosis and allow eosinophil survival under the influence of IL-5, IL-3 and GM-CSF (Simon and Alam, 1999). A study demonstrated that eosinophils exhibit prolonged survival depending on Lyn tyrosine Kinase phosphorylation (Simon *et al.*, 1998).

Caspases are a family of conserved cysteine proteases that play a major role in apoptosis (Pop and Salvesen, 2009, Salvesen, 2002). Functionally, caspases are classified into initiators (caspase 8, 9 and 10) and effectors (caspase 3, 6 and 7). These cysteine proteases are present within cells as inactive zymogens which need to be cleaved to generate free catalytic subunitsthat associate and form active heterotetramers to induce apoptosis (Fuentes, Prior and Salvesen, 2004, Park and Bochner, 2010, Salvesen, 2002). Eosinophils have been described to express caspases 3, 6, 7, 8 and 9, and these caspases are associated with spontaneous eosinophil apoptosis (Dewson *et al.*, 2001, Ilmarinen *et al.*, 2014). In addition, Bcl-2 family members are crucial to monitor intracellular damage and for mitochondrial membrane permeabilisation (MMP) in the intrinsic pathway of apoptosis. Several studies suggest that eosinophils contain small numbers of mitochondria, which are involved in the induction of apoptosis (Ilmarinen *et al.*, 2014, Park and Bochner, 2010, Tait and Green, 2010).

Many factors can significantly prolong eosinophil survival and delay spontaneous apoptosis, such as pathogens, cytokines (i.e TNF-α, leptin, interferons) and allergens (Ilmarinen *et al.*, 2014). In contrast, there is active apoptosis by Fas (CD95, APO-1), a cell surface receptor expressed on eosinophils as well as many other cells such as T cells and fibroblasts. Fas mediates apoptosis when ligated by agonistic antibodies or its natural ligand, FasL (Park and Bochner, 2010). Fas has been shown to be able to modify eosinophil survival and induce apoptosis of eosinophils (Matsumoto *et al.*, 1995). In addition, eosinophils apoptosis by Fas can be only partially overcome by IL-5 (Letuve *et al.*, 2001), and it can be further promoted by glucocorticoids (Druilleh *et al.*, 1996).

Glucocorticoids are pharmacological agents that are able to cause a marked reduction in eosinophil numbers *in vivo* (Schleimer, 1990). This effect can only be

overcome by high concentrations of IL-5 (Welsh and Wardlaw, 1997). Glucocorticoids accelerate eosinophil apoptosis and engulfment by lung macrophages (Megahaer *et al.,* 1996). They inhibit eosinophils to respond to GM-CSF (Lamas *et al.,* 1991) and inhibit cytokine-mediated eosinophil survival (Wallen *et al.,* 1991). Interestingly, horses with EG respond to steroid (glucocorticoid) therapy, but not horses with MCT (Kiupel, 2017). Furthermore, other agents can induce and mediate eosinophil apoptosis, including Siglec 8TGF-β (Alma *et al.,* 1994), CD30 (Matsumoto et al, 2004) and Lidocaine (Okada *et al.,* 2004).

Regardless of the mechanism involved, during apoptosis, eosinophils express phosphatidylserine on their plasma membrane which leads to their recognition and engulfment by macrophages. This process is important as the failure of macrophages to clear these cells may lead to secondary necrosis and the release of eosinophil granule proteins as well as other pro-inflammatory mediators (Farahi *et al.,* 2011).

Notably, in a study conducted in 1992 by Stern and colleagues about the programmed cell death of eosinophils, eosinophils were recognised and ingested as intact cells (phagocytosis) by macrophages *in vitro*. This study described the mechanism of apoptosis for aged human eosinophils but not fresh or aged pre-apoptotic eosinophils, which eventually leads to phagocytosis of the whole cell by macrophages. This process was regulated under the influence of IL-5. Furthermore, this study showed evidence that IL-5 plays a major role in supporting eosinophil survival but does not prevent the apoptosis mechanism completely. The researchers concluded that apoptosis and ingestion by macrophages could represent a mechanism whereby tissue longevity and removal of eosinophils is controlled (Stern *et al.,* 1992).

Interestingly, it was then shown that IL-5 enhances the phagocytosis by macrophages and that it also increases the ability of macrophages to kill bacteria. Such observations suggest that IL-5 augments the macrophage functions in MCT to engulf eosinophils (Linch *et al.,* 2012). These observations support the present finding that there is higher IL-5 production in MCT which increases eosinophil survival. However, the eosinophils will eventually die due to apoptosis, which leads

to recognition of the eosinophils and phagocytosis (ingestion) by macrophages as intact cells (Stern *et al.,* 1992).

4.2.12. Further analysis of eosinophils and EGC in Lendrum's stained sections

The present study demonstrated that Lendrum's stain highlights the eosinophil granules and also yielded different staining colours (purple, intense red, pale red) or colourless areas in EGC. In HE stained sections of EGC, the central necrotic granuloma core usually exhibits granular to amorphous eosinophilic material, which consists of intact eosinophils, eosinophils granules and nuclear debris, and entirely amorphous material (Scott and Miller, 2011a). However, no real difference in colour is appreciable.

The Lendrum's stain is used routinely in diagnostic laboratories to assess the presence of eosinophils (Bancroft and Gamble, 2008, Crocker and Burnett, 2005). It has been used effectively to count eosinophils in dental disease-associated syndromes such as TATE (tumour associated tissue eosinophilia) (Alkhabuli and High, 2006, Peter *et al.*, 2015, Vaibhav *et al.*, 2018). However, eosinophils in tissue usually have an amoeboid or "medusa" cell shape, especially in fibrous tissue or inflammatory infiltrates. It follows that their identification in routinely stained sections may be hard (Lorena *et al.,* 2003, Lowe *et al.,* 1981, Lendrum 1944). Lendrum's stains eosinophils selectively and intensely, which enhances the ease of counting (Peter *et al.,* 2015, Ladke Vaibhav *et al.,* 2018).

Surprisingly, the morphological evaluation of EG using Lendrum's stain demonstrated that the granuloma core does not follow a regular centrifugal development as expected, but an "irregular onion ring" like arrangement, with different orders of colour change, overlapping each other. Purple areas in Lendrum's stain overlapped perfectly with von Kossa positive areas. This result shows the capability of Lendrum's stain to demonstrate mineralisation. In the present study it identified mineralised necrotic material deriving from eosinophils.

In addition, in the present study, EGC areas in both EG and MCT exhibited different colours and yielded four patterns (purple, intense red, pale red and colourless). This leads to the hypothesis that eosinophil granules lose their affinity for Lendrum's

stain in a way that is proportional to their degree of degeneration. This hypothesis was supported by the fact that, despite the complex and onion-ring like arrangement, there was always a continuity between colours with the following sequence being noted: intense red - pale red - colourless, from the external portion to the core. When purple (mineralisation) was present, this tended to be at the periphery.

This result demonstrates that it is more likely that there will be mineralisation in true EG and on the other hand eosinophils intact or with fading/lacking granules (degenerating/degranulating) in MCT. The presence of more mineralisation in the core of EGC of Category I may suggest that there is more dystrophic mineralisation in those areas, possibly coming from eosinophils necrosis. In contrast, the increased intense red stain in MCT compared to EG coupled with higher numbers of infiltrating viable eosinophils may suggest the ability of neoplastic mast cells to keep eosinophils survival due to IL5 and eventually eosinophils dies by apoptosis.

4.3 Assessment of T cells, B cells and macrophages

4.3.1 T cells

CD 3 was used as a marker to identify T cells in the present study. T cells regulate adaptive immunity and are responsible for cell-mediated immune responses (Tizard, 2013, Day and Schultz 2014, Murphy and Weaver, 2017). Migrating lymphocytes are important regulators for efficient immune responses. T cell trafficking is the first and initial response. In this process, naïve T cells migrate through the specialised endothelium of secondary lymphoid organs and the primed T cells exert their function by infiltrating through capillary venules into the tissues and to their designated antigenic site (Oelkrug and Ramage, 2014, Carman and Martinelli, 2015). In addition, activation and differentiation of T cells into effector or memory lymphocytes induces the expression of specific receptors (Zhu *et al.*, 2010). The chemokine environment plays a pivotal role in controlling lymphocyte trafficking (Stein and Nombela-Arrieta, 2005). Chemokines are typically involved in the recruitment of lymphocytes, and their secretion by tissue has been proven to have an effect on the recruitment of certain T cells (Oelkrug and Ramage, 2014). Furthermore, macrophages express major histocompatibility complex (MHC) class II molecules on their surface, and present antigens to lymphocytes (Mantegazza *et*

al., 2013, Suzuki and Luo, 2017). When macrophages engulf a microbe, its antigens are processed and exposed on the outer surface of the plasma membrane where they will be recognised by T helper cells (Mantegazza *et al.*, 2013). At the site of chronic inflammation, both pro-inflammatory cytokines and chemokines work in coordination and attract mature eosinophils to the site (Barnes, 2008, Ghosh *et al.*, 2013). It is also the case that eosinophil recruitment and production is largely due to the stimulation of Th2 lymphocytes, as well as numerous cytokines (i.e. IL-3, IL-4, IL-5, IL13), and GM-CSF (Davoine and Lacy, 2014, Kita, 2013, Kita, 2011, Long *et al.*, 2016). The way of eosinophil trafficking to inflamed tissues is monitored by T cells responding to antigen presenting cells (Eng and Deflice, 2016).

Imunosurveillance by T cells is the fundamental process which the immune system uses for defence against cancer. However, T cell-mediated responses against tumour cells are usually slow and less efficient than responses against pathogen antigens (Govers, *et al.*, 2010).

The evaluation of the distribution of T cells (CD3-positive) in lesions of the five categories, demonstrated a high numbers of T cells in Category I lesions. These diminished to a minimum in Category V. T cells were higher in number in EG than in MCT. This result suggests more intense T cell recruitment into EGC of EG, and this is likely to be a consequence of different kinds of stimuli (exogenous and/or endogenous, as suggested by involvement of hair follicles and hitherto discussed). It is also possible that T cells are involved in eosinophil recruitment into EG more than into MCT. However, relevant numbers of T cells are also present in MCT and this suggests that T cells are involved in the cell crosstalk in both types of lesions, with a suspected major role in EG. In fact, interaction with eosinophils and mast cells is reported to play a major role in the modulation, activation and recruitment of T cells (Rothenberg and Hogan, 2006).

4.3.2 B cells

In this study, CD79a was used as a marker to identify B cells. B cells play a key role in inflammation and are essential in humoral and adaptive immunity (Chaplin, 2010). They are generated and matured in bone marrow and differentiate in the spleen and lymph nodes into naïve, follicular or marginal zone B cells (Pieper *et*

al., 2013). The marginal zone B cells are partially activated in conjunction with macrophages and dendritic cells, and exposed to antigens present in blood. In turn they provide a rapid humoral response to any challenges (Muppidi *et al.*, 2011, Cerutti *et al.*, 2013, Zouali and Richard, 2011). Marginal B cells contribute early in the adaptive response and following activation, and form short-lived plasma cells that secrete antibodies (Cerutti *et al.*, 2013, Hoffman *et al.*, 2016, Murphy and Weaver, 2017, Tizard, 2013, Zouali and Richard, 2011). B cells become active when they encounter an antigen (internalise and process). Thereafter they present it to a perfectly matched T-helper cell which then helps to activate the B cell (Hoffman *et al.*, 2016, Murphy and Weaver, 2017, Tizard, 2013). They are activated by many antigens, especially monomeric proteins. The process requires both binding of the antigen by the immunoglobulin on the B cell surface, the B-cell receptor, and interaction of the B cell with antigen-specific helper T cells (Avalos *et al.*, 2014, Murphy and Weaver, 2017). B cells are the sources of antibodies and also act as antigens to other effector cells, modulating antibody-mediated rejection (Lim *et al.*, 2015).

However, little is understood so far about B cell-eosinophil interaction and the ability of eosinophils to travel to the rich B cells areas has not, to date, been illustrated (Prussin *et al.*, 2013). Interestingly, a study in human and animal models *in vitro* demonstrated that eosinophils play a role to regulate the humoral immunity via their impact on B cell homeostasis and proliferation upon activation (Wong *et al.*, 2014).

The evaluation of the distribution of CD79a-positive B cells in the five categorise, demonstrated CD79a-positive B cells in only few lesions; these were almost exclusively EG (Category I). Some Category I lesions exhibited follicle-like B cell accumulations and a spatial relation of EGC to hair follicles. Such B cells could represent a component of the more generic response against the causative agent which leads to the formation of the EG. It is possible that there is no direct role for B cells in the genesis of the EGC, and that this represents the humoral part of the immune response generated by the causative agent.

4.3.3 Macrophages

In the present study, Iba1 was used as a marker to identify macrophages. The evaluation of the density of Iba1-positive macrophages in the five categorise demonstrated large numbers of macrophages infiltrating both EG and MCT. There was a slightly higher macrophage density in EG compared to MCT.

Macrophages play a role in granuloma formation. Macrophages are detected forming a palisading layer of cells surrounding the granuloma core (epithelioid macrophages) and are also responsible for the formation of inflammatory giant cells often associated with this type of inflammation (Hilhosret *et al.,* 2014). Presumably, difficult-to-eliminate antigens lead to the induction of monocytes and macrophages to form a complex granuloma structure based on activating signals from other cell populations (Williams and Williams, 1983). Granulomatous infiltrates reflect typical immune responses and are associated with inflammation and the potential for tissue destruction (Gordon, 2003). In inflammation, granuloma predominately consists of macrophages, and is based on the sources of the substances granulomatous diseases classified into: infectious granulomatous (i.e. Bacteria and fungi) and non-infectious granulomatous (i.e. keratin, suture and degenerated tissues) (Asai, 2017).

Granulomas structure may transform into necrotic or fibrotic tissues. However, the precise mechanism of progression and regulation is still unclear (Pagán and Ramakrishnan, 2018). Macrophages polarisation was found to be a key pathogenic factor in infectious granulomas in vivo and in vitro model studies which recognised that M1 macrophages promote granuloma formation and macrophage bactericidal activity, whereas (M2 macrophages) inhibit these effects (Huang *et al.,* 2015). Furthermore, macrophage polarisation suggested to maybe play a role in histopathogeneses in non-infectious granulomas (Barros *et al.,* 2013). Several studies demonstrated an active role of macrophages in eosinophils recruitment (Lukacs *et al.,* 1995).

When such roles are taken together, it can be concluded that macrophages are key players to form granuloma structures in infectious and non-infectious diseases and that many factors can influence their polarisation and immune responses. The

presence of macrophages was high in EGC and suggests that they have a major role in recruiting eosinophils to the site of inflammation.

Interestingly, in addition to the macrophages associated with the EGC, there was a large number of macrophages infiltrating between the neoplastic mast cells in MCT.

Macrophages are the most abundant inflammatory cells in tumours (tumour associated macrophages, TAMs), and are able to adapt to distinct functions and plasticity within tissues (Nielsen and Schmid, 2017, Noy and Pollard, 2014). According to existent literature, TAMs have tumour-supporting or anti-tumour functions (Ramanathan and Jagannathan, 2014, Parisi *et al.*, 2018).

In carcinogenesis, macrophages play a role in controlling the initial stage of cancer cell proliferation (i.e. carcinogenesis) cooperation with T lymphocytes (Vesely *et al.,* 2011). In contrast, the subverted macrophages or TAMs play a major role in dictating the progression of tumours through different mechanisms that vary according to the type of tissue, and inflammatory mediators (Yang and Zhang, 2017, Parisi *et al.*, 2018). Non-resolving chronic inflammation, which is a feature and marker of cancer, is associated with tumour microenvironment (TME) (Hanahan and Weinberg, 2011). This is considered to be a key element that links both chronic inflammation and the progression of cancer (Mantovani *et al.*, 2008, Ruffell and Coussens, 2015). Lymphocytes are the main regulator of the function and activity of tumour-associated macrophages (TAMs). However, the pathways are variable in different organs (Sica and Mantovani, 2012). For example, a switch of macrophage function was found to be mediated and influenced by IL-4 of Th2 cells in the skin (Andreu *et al.,* 2010, Schioppa *et al.,* 2011), and by antibody-producing B cells in breast cancer in humans (DeNardo *et al.,* 2009, Pedroza-Gonzalez *et al.,* 2011). Cancer progression can be promoted by B1 cells and macrophage polarisation whilst tumour promotion can be regulated by fibroblasts (Biswas and Mantovani, 2010). In addition, other cell products in tumour tissues can also reshape macrophages in an M2-like cancer mode due to extracellular matrix components, IL-10, CSF-1, and various chemokines (e.g. CCL2, CCL18, CCL17, and CXCL4) according to Sica and Mantovani (2012).

Macrophages express activities that can prevent the establishment and spread of tumour cells. They can also elicit functions to support tumour growth and dissemination. This paradoxical relationship between macrophages and tumour cells reflects the functional plasticity of these cells; they can express different functional programmes in response to different micro-environmental signals. The presence of macrophages in MCT suggests that they play a major role in preventing and/or initiating tumour growth through different mechanisms and inflammatory mediators as demonstrated in other neoplasms. It may also be the case that they play a role in eosinophils recruitment.

4.4 The role of cytokines and chemokines in the EG and MCT

The present and previous studies have shown that cutaneous MCT in horses are consistently infiltrated by high numbers of eosinophils (Hubert, 2006), and in some cases also by other leukocytes, i.e. lymphocytes (mainly T cells) and macrophages.They also showed that equine MCT are frequently associated with EG-like lesions (Scott and Miller, 2011c). This association suggests that one lesion (most likely the MCT) may trigger the development of the other (most likely the EGC). In the attempt to unravel this close relationship, the present study aimed to understand the pathogenesis and development of both MCT and EG, using an *in situ* approach based on the differential expressions of a range of cellular mediators (cytokines, chemokines and growth factors) that likely influence the interplay between eosinophils and other relevant cell populations in both entities (e.g. mast cells, lymphocytes, and macrophages).

Due to cost considerations and the often poor RNA preservation in older paraffin blocks, this part of the project was performed on a limited number of cases. This, nonetheless, enabled the discovery of some general patterns that may help our understanding of the pathogenesis of MCT and EG. In general terms (without differentiation between groups), RANTES was the marker expressed by the highest numbers of cells (up to 23 cells/HPF), followed by eotaxin (up to 2049 cells/cm^2), IL-13 (up to 777 cells/cm^2), IL-5 (up to 472 cells/cm^2) and MIP1α (up to 147cells/cm^2). In contrast, the number of cells that showed IL-4 transcription was very low (30 cells/cm^2), suggesting that this cytokine is not a main ruler in the cell interplay that occurs in these lesions.

SCF is a critical growth factor for MCs, regulating their activation, degranulation, survival, and chemotaxis (Meininger *et al.,* 1992). Several cell types can produce SCF (da Silva *et al.,* 2006), but within the lesions in the present study, only slender, often spindle shaped cells compatible with endothelial cells, fibroblasts and some vascular smooth muscle cells were found to express SCF mRNA (visible as faint dots) in all cases. Due to this generalised, obviously constitutive expression, it was not possible to attribute a specific role in either entity to this mediator.

TGF-β is a pleiotropic immunoregulatory cytokine that, for instance, antagonises the effects of IL-5 on eosinophils (Alam *et al.,* 1994), and exerts an important role in extracellular matrix protein deposition, particularly collagen (Davoine and Lacy, 2014). This was a feature that was also observed in the lesions in this study. In the present analysis, TGF-β was constitutively expressed by many cell types within the lesions. It was, again, not possible to attribute a specific role in either entity to this mediator.

RANTES/CCL5 has been described by, amongst others, Levy, (2009), and Appay and Rowland-Jones, (2001), as a highly pleiotropic chemokine that can be produced by many cell types (including T cells, macrophages, eosinophils and mast cells) with the potential to recruit a variety of leukocytes (including the same cells responsible for their production). In this study, RANTES was expressed by lymphocytes and some palisading macrophages in EGC, and at higher amounts in EG (Category I) compared to MCT (Categories III and IV), where only some scattered cells compatible with lymphocytes showed RANTES expression. This observation may partially contribute to the recruitment of different leukocytes in EG. However, the higher number of RANTES+ lymphocytes in EG may be explained by the higher number of T lymphocytes (CD3-positive) observed in this group and the fact that RANTES+ cells apparently account for a smaller subpopulation of the whole T cell repertoire (see Figure 3.51) .

Eotaxin-1/CCL11 can also be secreted by many cell types, including fibroblasts, certain epithelial cells, and leukocytes like macrophages, T cells, and mast cells (Rankin *et al.,* 2000). However, eotaxin is very well known as a potent and specific stimulator of eosinophil chemotaxis (Rankin *et al.,* 2000, Weston *et al.,* 2006). EG generally expressed a relatively abundant signal for eotaxin in cells compatible with

macrophages, fibroblasts and lymphocytes (with the exception of two of the five EG lesions that were tested, which were basically negative). Very rare or no eotaxin-positive cells were detected in the MCT, with the exception of some ulcerated MCT which showed the presence of neutrophil infiltrates. These MCT showed abundant roundish eotaxin-positive cells but also an important component of eotaxin-positive fibroblastoid cells (granulation tissue). There is some controversy regarding the effect of eotaxin on neutrophil recruitment, although there are some authors who suggest that eotaxin has an effect on neutrophil chemotaxis (Huaux *et al.,* 2005, Menzies-Gow *et al.,* 2002), whilst other reports have documented that there is no effect (Cheng *et al.,* 2002, Garcia-Zepeda *et al.,* 1996). This controversy led to the idea that it is not eotaxin that recruits those neutrophils and that there may be bacteria (as a result of the ulceration) that directly attract neutrophils, and indirectly recruit eosinophils possibly via eotaxin and IL-5 induction in nearby cells (Ravin and Loy, 2016).

MIP1α is the third chemokine included in this study that was expressed by scattered lymphocytes and macrophages. MIP1α was slightly more abundantly expressed in EG compared to MCT. However, the level of expression was generally very low and suggests that this chemokine is not a main ruler in either pathological condition. Th2 lymphocytes are known to generate the cytokines IL-4, IL-5 and IL13, which play important roles in regulating eosinophil recruitment (Rankin *et al.,* 2000). Despite the potential role of **IL-4** in eosinophil biology (Shakoory *et al.,* 2004, Rankin *et al.,* 2000) and the observation of some correlations with other mediators and cells in the statistical analysis, the extremely low number of cells expressing IL-4 mRNA in this study suggests that this cytokine is also not a main ruler.

IL-5 is the most specific cytokine regulating eosinophil development, playing a crucial role in driving committed eosinophil progenitor-cell proliferation, terminal differentiation and activation (Sanderson, 1990). In this study, IL-5 was expressed in numerous lymphocytes and mast cells in MCT, compared with the scattered positive lymphocytes observed in EG. While IL-5 does not appear to be nearly as important as chemokines for eosinophil recruitment, locally produced IL-5 does have an important function in increasing the survival of eosinophils once they have reached tissue (Park and Bochner, 2010, see also Chapter 4.9), suggesting an

explanation as to why we observed a certain association between the density of viable eosinophils within the MCA and IL-5 production (Figure 60).

Like IL-4, **IL-13** is a Th2 cytokine with relevant roles in eosinophil biology, inducing transendothelial eosinophil migration, direct eosinophil activation and attraction, and triggering production of eosinophil-active mediators (Shakoory *et al.*, 2004). In the present study, IL-13 was found to be highly expressed in EG and to a lesser extent in MCT. This suggests a major contribution not only in EG pathogenesis but also in MCT development. This finding supports the fact that IL-13 was mainly detected in T cells and that T cells (CD3-positive) are more abundant in EG whilst also being present in MCT. This in turn suggests that IL13-secreting T cells are involved in the crosstalk in both lesions, potentially playing a major role in EG. According to these findings, it could be hypothesised that IL-5 has a major role in maintaining viable eosinophils in MCT, and that there is a partial contribution by IL-13. However, in EG eotaxin may have a greater role in recruiting, whilst the scarcity of IL-5 expressing cells explains the lower viability of eosinophils. One possible mechanism in EG development includes an IL13-eotaxin-RANTES axis with the following interactions: (1) IL-13 stimulates VCAM-1 induction (Bochner *et al.*, 1995) and selective endothelial transmigration of eosinophils (Jia *et al.*, 1999), (2) IL-13 induces eosinophil active chemokines (i.e. eotaxin, RANTES) (Li *et al.*, 1999), recruiting eosinophils, T lymphocytes and macrophages, (3) IL13 directs activation, chemoattraction and survival of eosinophils (Horie *et al.*, 1997), and (4) IL-13 stimulates growth and differentiation of B cell aggregates (Murphy, 2012) which are frequently found in EG.

It is well described that eosinophils can secrete several of the molecules included in this study (Rothenberg and Hogan, 2006, Davoine and Lacy, 2014). However, we did not detect expression of any of our markers in eosinophils. Moreover, eosinophils also lacked signals even with housekeeping gene control probes such as PPIB and UBC. This observation may be attributable to the high density of eosinophil granules in the cells which might obstruct the binding of the probes to target mRNA.

4.5. Conclusion

This study attempted to classify EG and MCT with a rigorous approach based on the relative amount of EGC and MCA within lesions. Pure EG lacked any area of MCA, while MCT variably presented EGC. EG and MCT differed for several morphological characteristics and when comparing the two lesions the main features found were as follows:

- EG were present in large numbers t in the most superficial areas of the dermis and often associated with hair follicles while MCT were not.
- EGC areas within EG were characterised by collagen fragments and mineralisation. This feature was not prominent in MCT.
- Lymphocyte aggregates were often localised within or in close proximity to the lesions in EG. They were not a feature of MCT.
- Macrophages, despite increased in EG, represented the predominant inflammatory cells in both EG and MCT.
- Macrophages were observed engulfing eosinophils in MCT, but not in EG.
- T cells were more represented in EG compared to MCT. However, it should also be noted that they were present in large numbers in MCT.
- B cells were scarce; they were evident only in EG.
- In EG eosinophils recruitment seems to be driven by different immune cells (lymphocytes and macrophages) secreting predominantly Rantes, IL13 and Eotaxin. MCT mast cells and potentially lymphocytes seem to possibly recruit eosinophils via secretion of IL-5 which also increases the survival of these cells.

These morphological and biological features differentiate EG from MCT and demonstrates that pure EG differs substantially from MCT. This supports the hypothesis that EG and MCT are two different entities with different pathogenesis.

APPENDIX I: METHODS

Histology standard operating procedure

General PPE requirements

- Howie Style Lab Coat
- Bodyguards nitrile gloves to BS EN455:2000 and BS EN374:2003
- Safety glasses BS EN166
- Eye protection: Face Shield BS EN 166-3B or F
- Respirator mask EN149:2001 & A1:2009 FFP3 NR

Reagents

- Formalin: Buffered Formalin Concentrate (Atom Scientific, RRFF5000-G). Contains Formaldehyde <50% (19-20% v/v), Mixed Phosphates. Add 100ml Buffered Formalin Concentrate to 400ml tap water to make working solution of 10% Formalin (1 in 5 dilution)
- 4% Paraformaldehyde (PFA): Due to toxic fumes make this solution up in a fume hood-dissolve 10g paraformaldehyde in 200ml PBS at 60^0C on stirrer (do not increase temperature over 70^0C as solution will precipitate). Adjust pH to 7.24-7.4. Add PBS to 250ml. Store for maximum of 1 month
- Ultraplast Polyisobutylene Histological Wax (Solmedia WAX060)
- Mayers Haematoxylin: 1g Haematoxylin 95% Monohydrate (Atom Scientific, cat no: RRBD61-X), 0.2g sodium iodate, 50g potassium or ammonium alum, 20ml glacial acetic acid, 1 litre distilled water. Dissolve haematoxylin in the water using gentle heat. Add potassium alum and dissolve using gentle heat. Cool and add sodium iodate. Mix and filter the solution. Add 20ml glacial acetic acid.
- Eosin Y stain: 50ml 1% Eosin Y (TCS Biosciences Ltd, cat no HS250-1L), 390ml ethanol, 2ml glacial acetic acid
- DPX mounting medium (Thermo Scientific, ref LAMB/DPX)

Appendix I

Method - Tissue handling

1. Fix tissue by immersion in 10% Neutral Buffered Formalin (or 4% PFA*) for 24 hours in a sealed pot (*better for immunohistochemistry procedures)
2. In a well ventilated room, remove tissue from fixative and cut to appropriate size/orientation & place within labelled plastic cassettes. Snap cassette closed using cassette lid. Very small tissue pieces may be held within a cassette by placing the tissue between two pieces of embedding sponge within the plastic cassette.
3. Process tissue within plastic cassettes on tissue processor overnight

Method – Tissue processing using TISSUE-TEK©VIP, Miles Scientific

Tissue processor removes water from tissue & replaces it with paraffin wax which penetrates and solidifies tissue and enables it to be cut. Consists of:

1. Tissue dehydration through immersion in increasing concentrations of ethanol
2. Immersion in xylene (removes ethanol)
3. Tissue infiltration with molten paraffin wax (replaces xylene)

Method – Tissue embedding and cutting sections

1. Place tissue in metal moulds containing molten paraffin wax. Gently press labelled plastic cassette on top of mould, and place on cold platform to solidify the wax block.
2. Remove tissue block from mould and trim off excess wax from sides of cassette. Place block in microtome and trim face of block until the full tissue section is being cut. Remove the block from microtome and place on ice for 30 minutes to cool before cutting.
3. Cut 4 micron sections on microtome, float sections in 45^0C water bath and collect onto glass slides.
4. Dry the cut sections at 37^0C for 30 minutes to allow adherence to slide before staining.

Appendix I

Method – Tissue staining

H&E for basic histological analysis as follows (carry out in fume hood):

1. Dewax sections in xylene 5 minutes
2. Transfer sections to another xylene and take sections down through descending grades of alcohol (100%, 96%, 85%, 70%) to distilled water
3. Stain sections in Mayers haematoxylin 5 minutes
4. "Blue" sections in running water 6 minutes
5. Stain sections in Eosin for 2 minutes
6. Take sections through 3x 96% alcohol for 1 minute each (dipping sections up and down to remove excess stain)
7. Take sections through 3x 100% alcohol
8. Take sections through 2x xylene
9. Mount sections in DPX mounting medium and place glass coverslip over top. Press out air bubbles using mounted needle by gently pressing on top of coverslip. Allow mounted sections to dry in fume cabinet.

Toluidine Blue Reagent

1% Toluidine Blue in distilled water (made in house from Toluidine Blue, 9756880F, BDH Chemicals Ltd, Poole, UK)

0.2% Acetic Acid in distilled water (made in house from Glacial Acetic Acid, A/0360/PB17, Fisher Scientific UK, Loughborough, UK)

Lendrum's method for eosinophils solutions

Carbol Chromatrope

Ready to use Lendrum's Chromotrope (641550, Clin-Tech Limited, Guildford, UK)

Mayers Haematoxylin

1g Haematoxylin 95% Monohydrate (RRBD61-X, Atom Scientific, Manchester, UK)

0.2g Sodium iodate (201761000, Acros Organics, New Jersey, USA)

Aluminium Potassium Sulphate (217485000, Acros Organics, New Jersey, USA)

1000mls distilled water

20mls glacial acetic acid (A/0360/PB17, Fisher Scientific UK, Loughborough, UK)

Masson Trichrome Solutions (all made in house)

Celestine Blue Solution

2.5g Celestine Blue (206342, Sigma-Aldrich, St Louis, USA)

25g Ferric Ammonium Sulphate (271644, BDH, Pool, UK)

70ml Glycerol (24388.295, VWR International, Fontenaysous- Bois)

500ml distilled water

Cytoplasmic Stain

Mix together:

2 parts 1% Ponceau de Xylidine (HD1800, HD Supplies, Aylesbury, UK)

1 part 1% acid fuchin (RRBD93/X, Biostain ready reagents, Manchester, UK) in 1% acetic acid (made in house from Glacial Acetic Acid, A/0360/PB17, Fisher Scientific UK, Loughborough, UK)

1% Phosphomolybidic acid

(206381000, Acros Organics, New Jersey, USA) made in house

2% Light Green

(HD1535, HD Supplies, Aylesbury, UK) made in house

Mayers Haematoxylin

1g Haematoxylin 95% Monohydrate (RRBD61-X, Atom Scientific, Manchester, UK)

0.2g Sodium iodate (201761000, Acros Organics, New Jersey, USA)

Aluminium Potassium Sulphate (217485000, Acros Organics, New Jersey, USA)

1000mls distilled water

20mls glacial acetic acid (A/0360/PB17, Fisher Scientific UK, Loughborough, UK)

Appendix I

A modification of Von Kossa's method for calcium solutions

5% silver nitrate in distilled water (made in house from Silver Nitrate, S/1280/46, Fisher Scientific UK, Loughborough, UK)
1% Neutral Red in 1% Acetic Acid (RRSP160-F, Atom Scientific, Manchester, UK)

Perls' Prussian Blue solutions

2% Potassium ferrocyanide in distilled water (102054F, VWR International Ltd, Poole, UK)
2% Hydrochloric acid in distilled water (H/1200/PB17, Fisher Scientific UK, Loughborough, UK)
1% Neutral Red in 1% Acetic Acid (RRSP160-F, Atom Scientific, Manchester, UK)

TBST buffer

10x TBS STOCK
66.57g Tris-(hyroxymethyl) aminomethane (28811.295, VWR International, Leuven, Belgium)
610ml distilled water
390ml 1N Hydrochloric Acid (made in house from H/1200/PB17, Fisher Scientific UK, Loughborough, UK) pH 7.6

TBST working solution

7.2g NaCl (59888, Sigma Aldrich, Steinheim, Germany)
100ml 10x TBS Stock
900ml distilled water
500µl Tween (P1379, Sigma Aldrich, Steinheim, Germany)

EDTA solution for pressure cooker 10x solution (1L)

Dissolve in 900ml (warm at 60ºC)
12gr Tris Base (Roche, 10708976001)
3.7gr EDTA (Ethylene diamine-tetra acetic acid) (Fluka, 03610)
Adjust with NaOH 1N at pH 9

Complete up to 1000ml

Dilute 1:10 before use

Immunohistochemistry protocol

CD3 protocol on Autostainer Link 48

Polyclonal Rabbit anti Human CD3 (Dako A0452)

PT Link

Deparaffinisation, rehydration and epitope retrieval using:

EnVision™ FLEX Target Retrieval Solution (TRS) High pH (Citrate buffer, pH 9.0, K8004)

Dako Autostainer Link 48

Step	Reagent	Volume	Time
Rinse	Buffer[1]		
Endogenous Enzyme Block	FLEX Peroxidase Block[2]	150µl	5 min
Rinse	Buffer[1]		
Primary Antibody	CD3 1:500 in diluent[3]	150µl	20 min
Rinse	Buffer[1]		
Labelled Polymer	FLEX/HRP[8]	150µl	20 min
Rinse	Buffer[1]		
Rinse	Buffer[1]		
Substrate-Chromogen	FLEX DAB+ Sub-Chromo[5+6]	150µl	5 min
Substrate-Chromogen	FLEX DAB+ Sub-Chromo[5+6]	150µl	5 min
Rinse	Buffer[1]		
Counterstain	FLEX Hematoxylin[7]	150µl	5 min
Rinse	DI Water		
Rinse	Buffer[1]		
Rinse	DI Water		

Appendix I

Autostainer set up to deliver 2x reagent drops of 150µl to upper and lower zones on the slide (position of reagent drops can be maximum of 3x zones per slide).

Following end of protocol remove slides to staining racks and dehydrate (1minute 96% ethanol, 2x 3minutes 100% ethanol), coverslip and mount in DPX.

Dako/AgilentReagents

1. EnVision™ FLEX Wash Buffer (K8007)
2. EnVision™ FLEX Peroxidase-Blocking Reagent (SM801)
3. EnVision™ FLEX Antibody Diluent (K8006)
4. Envision™+System-HRP Labelled Polymer α Rabbit (K4003)
5. EnVision™ FLEX DAB+ Chromogen (DM827)
6. EnVision™ FLEX Substrate Buffer (SM802)
7. EnVision™ FLEX Hematoxylin (K8008)
8. EnVision™ FLEX/HRP (SM802)

CD79a protocol on Autostainer Link 48

Monoclonal mouse anti-human CD79a (AbDserotec,MCA2538H)

PT Link

Deparaffinisation, rehydration and epitope retrieval using:
EnVision™ FLEX Target Retrieval Solution (TRS) Low pH (Citrate buffer, pH 6.1, K8005)

Dako Autostainer Link 48

Step	Reagent	Volume	Time
Rinse	Buffer[1]		
Endogenous Enzyme Block	FLEX Peroxidase Block[2]	150µl	5 min
Rinse	Buffer[1]		
Primary Antibody	CD79a 1:300 in diluent[3]	150µl	20 min
Rinse	Buffer[1]		
Labelled Polymer	FLEX/HRP[8]	150µl	20 min
Rinse	Buffer[1]		
Rinse	Buffer[1]		
Substrate-Chromogen	FLEX DAB+ Sub-Chromo[5+6]	150µl	5 min

Substrate-Chromogen	FLEX DAB+ Sub-Chromo[5+6]	150µl	5 min
Rinse	Buffer[1]		
Counterstain	FLEX Hematoxylin[7]	150µl	5 min
Rinse	DI Water		
Rinse	Buffer[1]		
Rinse	DI Water		

Autostainer set up to deliver 2x reagent drops of 150µl to upper and lower zones on the slide (position of reagent drops can be maximum of 3x zones per slide).

Following end of protocol remove slides to staining racks and dehydrate (1minute 96% ethanol, 2x 3minutes 100% ethanol), coverslip and mount in DPX.

Dako/AgilentReagents

1. EnVision™ FLEX Wash Buffer (K8007)
2. EnVision™ FLEX Peroxidase-Blocking Reagent (SM801)
3. EnVision™ FLEX Antibody Diluent (K8006)
4. Envision™+System-HRP Labelled Polymer α Rabbit (K4003)
5. EnVision™ FLEX DAB+ Chromogen (DM827)
6. EnVision™ FLEX Substrate Buffer (SM802)
7. EnVision™ FLEX Hematoxylin (K8008)
8. EnVision™ FLEX/HRP (SM802)

C-Kit protocol on Autostainer Link 48

Polyclonal mouse anti-human CD117 (Dako,A4502)

PT Link

Deparaffinisation, rehydration and epitope retrieval using:

EnVision™ FLEX Target Retrieval Solution (TRS) High pH (Citrate buffer, pH 9.0, K8004)

Appendix I

Dako Autostainer Link 48

Step	Reagent	Volume	Time
Rinse	Buffer[1]		
Endogenous Enzyme Block	FLEX Peroxidase Block[2]	150µl	5 min
Rinse	Buffer[1]		
Primary Antibody	C-kit 1:500 in diluent[3]	150µl	20 min
Rinse	Buffer[1]		
Labelled Polymer	FLEX/HRP[8]	150µl	20 min
Rinse	Buffer[1]		
Rinse	Buffer[1]		
Substrate-Chromogen	FLEX DAB+ Sub-Chromo[5+6]	150µl	5 min
Substrate-Chromogen	FLEX DAB+ Sub-Chromo[5+6]	150µl	5 min
Rinse	Buffer[1]		
Counterstain	FLEX Hematoxylin[7]	150µl	5 min
Rinse	DI Water		
Rinse	Buffer[1]		
Rinse	DI Water		

Autostainer set up to deliver 2x reagent drops of 150µl to upper and lower zones on the slide (position of reagent drops can be maximum of 3x zones per slide).

Following end of protocol remove slides to staining racks and dehydrate (1minute 96% ethanol, 2x 3minutes 100% ethanol), coverslip and mount in DPX.

Dako/AgilentReagents

1. EnVision™ FLEX Wash Buffer (K8007)
2. EnVision™ FLEX Peroxidase-Blocking Reagent (SM801)
3. EnVision™ FLEX Antibody Diluent (K8006)
4. Envision™+System-HRP Labelled Polymer α Rabbit (K4003)
5. EnVision™ FLEX DAB+ Chromogen (DM827)

6. EnVision™ FLEX Substrate Buffer (SM802)
7. EnVision™ FLEX Hematoxylin (K8008)
8. EnVision™ FLEX/HRP (SM802)

C-Kit protocol on Autostainer Link 48

Polyclonal mouse anti-human CD117 (Dako,A4502)

PT Link

Deparaffinisation, rehydration and epitope retrieval using:

EnVision™ FLEX Target Retrieval Solution (TRS) High pH (Citrate buffer, pH 9.0, K8004)

Dako Autostainer Link 48

Step	**Reagent**	**Volume**	**Time**
Rinse	Buffer[1]		
Endogenous Enzyme Block	FLEX Peroxidase Block[2]	150µl	5 min
Rinse	Buffer[1]		
Primary Antibody	Iba1 1:500 in diluent[3]	150µl	20 min
Rinse	Buffer[1]		
Secondary Reagent	anti-goat IgG[9]	150µl	20 min
Rinse	Buffer[1]		
Rinse	Buffer[1]		
Substrate-Chromogen	FLEX DAB+ Sub-Chromo[5+6]	150µl	5 min
Substrate-Chromogen	FLEX DAB+ Sub-Chromo[5+6]	150µl	5 min
Rinse	Buffer[1]		
Counterstain	FLEX Hematoxylin[7]	150µl	5 min
Rinse	DI Water		
Rinse	Buffer[1]		
Rinse	DI Water		

Appendix I

Autostainer set up to deliver 2x reagent drops of 150µl to upper and lower zones on the slide (position of reagent drops can be maximum of 3x zones per slide).

Following end of protocol remove slides to staining racks and dehydrate (1minute 96% ethanol, 2x 3minutes 100% ethanol), coverslip and mount in DPX.

Dako/AgilentReagents

1. EnVision™ FLEX Wash Buffer (K8007)
2. EnVision™ FLEX Peroxidase-Blocking Reagent (SM801)
3. EnVision™ FLEX Antibody Diluent (K8006)
4. Envision™+System-HRP Labelled Polymer α Rabbit (K4003)
5. EnVision™ FLEX DAB+ Chromogen (DM827)
6. EnVision™ FLEX Substrate Buffer (SM802)
7. EnVision™ FLEX Hematoxylin (K8008)
8. EnVision™ FLEX/HRP (SM802)
9. ImmPRESSTM HRP Reagent Kit Peroxidase anti-Goat IgG (MP-7405)

MCT protocol on Autostainer Link 48

Monoclonal mouse anti-human Mast Cell Tryptes (Dako,M7052)

PT Link

Deparaffinisation, rehydration and epitope retrieval using:
EnVision™ FLEX Target Retrieval Solution (TRS) Low pH (Citrate buffer, pH 6.1, K8005)

Dako Autostainer Link 48

Step	Reagent	Volume	Time
Rinse	Buffer[1]		
Endogenous Enzyme Block	FLEX Peroxidase Block[2]	150µl	5 min
Rinse	Buffer[1]		
Primary Antibody	MCT 1:500 in diluent[3]	150µl	20 min
Rinse	Buffer[1]		
Labelled Polymer	FLEX/HRP[8]	150µl	20 min
Rinse	Buffer[1]		
Rinse	Buffer[1]		

Substrate-Chromogen	FLEX DAB+ Sub-Chromo[5+6]	150µl	5 min
Substrate-Chromogen	FLEX DAB+ Sub-Chromo[5+6]	150µl	5 min
Rinse	Buffer[1]		
Counterstain	FLEX Hematoxylin[7]	150µl	5 min
Rinse	DI Water		
Rinse	Buffer[1]		
Rinse	DI Water		

Autostainer set up to deliver 2x reagent drops of 150µl to upper and lower zones on the slide (position of reagent drops can be maximum of 3x zones per slide).

Following end of protocol remove slides to staining racks and dehydrate (1minute 96% ethanol, 2x 3minutes 100% ethanol), coverslip and mount in DPX.

Dako/AgilentReagents

1. EnVision™ FLEX Wash Buffer (K8007)
2. EnVision™ FLEX Peroxidase-Blocking Reagent (SM801)
3. EnVision™ FLEX Antibody Diluent (K8006)
4. Envision™+System-HRP Labelled Polymer α Rabbit (K4003)
5. EnVision™ FLEX DAB+ Chromogen (DM827)
6. EnVision™ FLEX Substrate Buffer (SM802)
7. EnVision™ FLEX Hematoxylin (K8008)
8. EnVision™ FLEX/HRP (SM802)

Appendix I

Lysozyme protocol on Autostainer Link 48

Poloclonal Rabbit anti-human Lysozyme (Dako,A0009)

PT Link

Deparaffinisation, rehydration and epitope retrieval using:
EnVision™ FLEX Target Retrieval Solution (TRS) Low pH (Citrate buffer, pH 6.1, K8005)

Dako Autostainer Link 48

Step	Reagent	Volume	Time
Rinse	Buffer[1]		
Endogenous Enzyme Block	FLEX Peroxidase Block[2]	150µl	5 min
Rinse	Buffer[1]		
Primary Antibody	Lysozyme 1:2500 in diluent[3]	150µl	20 min
Rinse	Buffer[1]		
Labelled Polymer	FLEX/HRP[8]	150µl	20 min
Rinse	Buffer[1]		
Rinse	Buffer[1]		
Substrate-Chromogen	FLEX DAB+ Sub-Chromo[5+6]	150µl	5 min
Substrate-Chromogen	FLEX DAB+ Sub-Chromo[5+6]	150µl	5 min
Rinse	Buffer[1]		
Counterstain	FLEX Hematoxylin[7]	150µl	5 min
Rinse	DI Water		
Rinse	Buffer[1]		
Rinse	DI Water		

Autostainer set up to deliver 2x reagent drops of 150µl to upper and lower zones on the slide (position of reagent drops can be maximum of 3x zones per slide).

Appendix I

Following end of protocol remove slides to staining racks and dehydrate (1minute 96% ethanol, 2x 3minutes 100% ethanol), coverslip and mount in DPX.

Dako/AgilentReagents

1. EnVision™ FLEX Wash Buffer (K8007)
2. EnVision™ FLEX Peroxidase-Blocking Reagent (SM801)
3. EnVision™ FLEX Antibody Diluent (K8006)
4. Envision™+System-HRP Labelled Polymer α Rabbit (K4003)
5. EnVision™ FLEX DAB+ Chromogen (DM827)
6. EnVision™ FLEX Substrate Buffer (SM802)
7. EnVision™ FLEX Hematoxylin (K8008)
8. EnVision™ FLEX/HRP (SM802)

PCNA Envision IHC Method

Steps of Envision (use separate rack)

Xylene 2x 5minutess

100% ethanol 2x 2minutes

96% ethanol 1x 2minutess

80% ethanol 1x 2minutess

70% ethanol 1x 2minutes

Leave in dH_20

Pre-treatments (Antigen Retrieval) Citrate 4

Rack up slides in sequenza clips using TBST (TBS Buffer + 0.05% Tween 20)

3x 2minute TBST wash

Peroxidase Block ~100µl DAKO REAL™ Peroxidase Blocking Solution S2023 (250ml) 10mins

3x 2minute TBST wash

~100µl Primary Antibody (PCNA, Dako, M0879) dilute 1:100 in TBST
4°C overnight

3x 2minute TBST wash

Apply 3 drops of Dako Envision+ System-HRP α mouse (K4001, 110ml)

30mins, room temp

3x2min TBST wash

Remove from sequenza clips to dH_20

DAB 10mins

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0.2g Diaminobenzidine in 400mls Imidazole Buffer:

(280ml 0.1M Imidazole/120ml 0.42MHCl pH 7.2), filtered, activate with 140µl 30% Hydrogen Peroxide H_20_2 (Fisher Scientific BP2633-500)

3x 5minute washes dH_20

Counterstain with Papanicolaou's 1b Hematoxylin Solution (Merck 1.09254.0500) 20mls in 400mls dH_20 stain 1minute.

"blue" running tap water 5 minutes

Dehydrate, Clear & Mount:

96% ethanol 1 minute

100% ethanol 2 minutes

100% ethanol 3 minutes

Xylene 2 minutes

Xylene 3 minutes

Xylene 3 minutes

Coverslip with DPX

The mRNA sequences of the selected chemokines/cytokines form NCBI

Equus caballus peptidylprolyl isomerase B (PPIB), mRNA

NCBI Reference Sequence: NM_001099761.1

>NM_001099761.1 Equus caballus peptidylprolyl isomerase B (PPIB), mRNA

TTCTCCCGGTGGATGCTGCGTTTCTCAGAGCGGAACATGAAGGTGCTC
TTCGCCGCCGCCCTCATCGTGGGCTCTGTCTTCTTCCTGCTGTTGCCAG
GACCCTCCACGGCCGATGAGAAGAAGAAGGGGCCTAAAGTCACTGTC
AAGGTGTACTTTGACCTGCGAATTGGAGATGAAGATATAGGCCGGGTG
GTCATCGGTCTCTTTGGAAAGACTGTTCCAAAAACAGTGGATAATTTT
GTGGCCTTAGCTACAGGAGAGAAAGGATTTGGCTACAAAGACAGCAA
ATTCCATCGTGTGATCAAGGACTTCATGATCCAGGGTGGAGACTTCAC
CCGGGGAGATGGCACTGGAGGTAAGAGCATCTACGGTGAACGCTTCC
CAGACGAGAACTTCAAGCTGAAACACTATGGGCCCGGCTGGGTGAGC
ATGGCCAACGCAGGCAAAGACACCAACGGCTCCCAGTTCTTTATCACG
ACCGTGAAGACAGCCTGGCTAGATGGCAAGCACGTAGTGTTCGGCAA
AGTTCTAGAGGGCATGGAGGTAGTGCGGAAGGTGGAGACCACCAAGA
CAGATGGGCGCGACAAGCCCCTGAAGGATGTGACAATTGCAGACTGT

GGCAAGATCGAGGTGGAGAAGCCCTTTGCCATCGCCAAGGAGTAGGG
CCTGGGGACTTCCTCCCTTTGAGCAACCATCTGTGCAGCCGTGTTGCCC
CCCAAGGGGTGAAGACAGCCTGCCACAGGGCTCTGCGCTCCCACTGGC
CCCAGTGGTGGCATCTGACGGAGTGGACTCCTCCCCTCACATTCCACA
GGGCCCAGTTTTGTAACAAACTCCTACCAACACTGACCAATAA

Equus caballus chemokine (C-C motif) ligand 3 (CCL3), mRNA

NCBI Reference Sequence: NM_001114941.1

>NM_001114941.1 Equus caballus chemokine (C-C motif) ligand 3 (CCL3), mRNA

ACTCCATCCGCTCAGCATCATGAAGGTCCCCGTGGCTGCCCTTGCCGT
CCTCCTCTGCACCATGGCCCTCTGCAGCCAGGTCTTCTCTGTACCATTC
GGTGCCGACACCCCAACTGCCTGCTGCTTCTCCTACGTCTCCCGGCAG
ATTCCGCGCAAATTCATAAACGACTATTATGAGACCAGCAGCCAGTGC
TCCAAGCCAGCCATCATCTTCCAAACCAAAAGAAGCCGGCAGGTCTGT
GCCGACCCCAGTGAGGCCTGGGTCCAGGAGTACGTGACCGACCTGGA
GCTGAGCGCCTGAGTGGCCAGTGACCTCGGCAGGCTGCCTGGAGCAC
AGGGCTGGGCCTTAGAAACAGCCTCGTAACCT

Equus caballus chemokine (C-C motif) ligand 11 (CCL11), mRNA

NCBI Reference Sequence: NM_001081871.1

>NM_001081871.1 Equus caballus chemokine (C-C motif) ligand 11 (CCL11), mRNA

AGAGCAGCAGAGACCAGCCCAGAAACCAACAGCTCTCACGCTGAAGC
TCGCGTCCTCGCCCTCCAGCATGAAGGTCTCCGCAGCCCTCCTGTGCCT
GCTGCTCACCACGGCCGCCTTCAGCACCCAGGTGCTGGCTCAGCCAGT
TTCTATCTCGACCGTCTGCTGCTTTAACGTGGCCAGTAGGAAGATCTCT
TTTCAGCGACTGCAGAGCTACAGAAAAATCACCAGCAGCAAATGTCCC
CAGAAAGCTGTGATCTTCAAGACCAAACAAGCCAAGAAGATCTGTGCT
GATCCCAAGCAGAAGTGGGTCCAGGATGCCATGAAGTACCTGGACGA
AAACTCCCGAACTACAAAGTATTCATCTTTTTGAGACCAAATCAGAGC
CAGAGGAATGCCTGATTCATCTTCCCTGCTCTTCCTAAGATGTGTCCTG
AGATAATTTCATCATCATTACAAAAGGAATGGCTTTTATTTAATAATTA
AAAAATAC ATATATTGCAAAAAAAAAAAAAA

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Equus caballus KIT ligand (KITLG), mRNA

NCBI Reference Sequence: NM_001163962.1

>NM_001163962.1 Equus caballus KIT ligand (KITLG), mRNA

CCGCCTCGCGCCGGGACCGGGAGCGCCGCGGGCATCACGGGCCGGAG
AGAGGGCGCTGCGCTCGGGCTGCCCAATGCGAATCCTATCTGCAGCCG
CTGCTCGTGCGGTATGCTGGAGCTCCAGAACAGCTAAACGCAGTCGCC
ACACCGCTGCCTGGGCTGGATCGCAGCGCTGCCTTTCCTTATGAAGAA
GACACAAACTTGGATTATCACTTGCATTTATCTTCAACTGCTCCTATTT
AATCCTCTCGTCAAAACCAAAGGAATCTGTGAAAACCGTGTGACTGAT
GATGTGAAAGACGTGACAAAATTGGTGGCAAATCTTCCAAAAGACTAT
AAGATAACCCTCAAATATGTCCCCGGGATGGACGTTTTGCCTAGTCAT
TGTTGGATAAGCGAGATGGTGCAACACTTGTCAGTCAGCTTGACTGAT
CTCTTGGAGAAGTTTTCAAATATTTCTGAAGGCTTGAGTAATTATTCTA
TCATAGACAAACTTGTGAAAATAGTGGATGATCTTGTGGAGTGCATGG
AAGAACACTCATCTGAGAATGTAAAAAAATCATATAAGAGCCAAGAA
TCCAGGCTATTTACTCCTGAAGAATTCTTTAGAATTTTTAATAGATCCA
TCGATGCCTTCAAGGACTTGGAGATGGTGGTATCTAAAACTAGTGAAT
GTGTGGTGTCTTCAACATTAAGTCCTGAAAAAGATTCCAGAGTCAGTG
TCACAAAACCATTTATGTTACCCCCTGTTGCAGCCAGCTCCCTTAGGAA
TGACAGCAGTAGCAGTAATAGGAAGGCCTCAAATTTCACTGGAGACTC
CAACCTACAATGGGCAGCCATGGCATTGCCAGCATTCTTTTCTCTTGTA
ATTGGGTTTGCTTTTGGAGCCTTATACTGGAAGAAGAAACAACCAAAT
CTTACAAGGGCAGTTGAAAATATACAGATTAACGAAGAGGATAATGA
GATAAGTATGTTGCAAGAAAAAGAGAGAGAGTTTCAAGAAGTGTAAT
TGTGGCTTGTGTCAACACTGTTACTTTCATACATTGGCGGGTAACAGTT
CATGTTTGCTTCATAAATGAAGCAGCTTTAAACAAATTCATATTCTGTC
TGGAGTGACAGACTGCATCTTTATCTGTTCTTGCTACCCACAACTCTGT
ATGGATGATTCAGAAATTGGAACAAAGTATTTTACTGTGAAACTGGCA
CTGAATTAATCATCTGTAAAGAAGAAGTTGCATGGAGCAGGACTCTAT
TTTAAGACCTGGGGGACTTGTGGTCTCATTTAGAACTTATAACTGATGT
TGGAAAAGAAAGCATGTGTTCCAGACTGCATGCACCATCTTGCATGCC
TCCAGAAATGTCTAATTACTGAAAAGCCACATAGCTTTATTTTCAGTTA
CAACCGTG

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Equus caballus interleukin 5 (IL5), mRNA

NCBI Reference Sequence: NM_001082499.1
>NM_001082499.1 Equus caballus interleukin 5 (IL5), mRNA
ATGAGGATGCTTCTGCATTTGAGTGTGCTAGCTCTTGGAGCTGCCTAC
GTCTGTGCCCTTGCTGTAGAAAGTCCCATGAACAGACTAGTGGCAGAG
ACCTTGACACTGCTCTCCACTCATCGAACTCTGCTGATAGGCGATGGG
AACCTGATGATTCCTACTCCTGAACATAAAAATCACCAACTCTGCATT
GAAGAAGTCTTTCAGGGAATAGACACATTGAAGAATCAAACTGTCCA
AGGGGATGCTGTGGCCAAACTATTCCAAAACTTGTCTTTAATAAAAGG
ATACATAGACCTCCAAAAAAAAAAGTGTGGAGGAGAAAGATGGAGAG
TGAAACAATTCCTAGACTACCTGCAGGAGTTTCTTGGTGTAATAAACA
CTGAGTGGACAATAGAAGGCTGA

Equus caballus interleukin 4 (IL4), mRNA

NCBI Reference Sequence: NM_001082519.1
>NM_001082519.1 Equus caballus interleukin 4 (IL4), mRNA
ATGGGTCTCACCTACCAACTGATTCCAGCTCTGGTCTGCTTACTAGCAT
GTACCAGCAACTTCATCCAGGGATGCAAATACGACATCACCTTACAAG
AGATCATCAAAACGCTGAACAACCTCACAGATGGAAAGGGCAAGAAT
TCGTGCATGGAGCTGACTGTAGCGGATGCCTTTGCTGGCCCGAAGAAC
ACAGATGGAAAGGAAATCTGCAGGGCTGCAAAGGTGCTTCAACAGCT
CTATAAAAGACATGACAGGTCCTTGATCAAAGAATGCCTGAGCGGACT
GGACAGGAACCTCAAGGGCATGGCAAACGGGACCTGCTGTACTGTGA
ATGAAGCCAAGAAGAGCACATTGAAAGACTTTTTGGAAAGGCTAAAG
ACGATCATGAAAGAGAAATACTCCAAGTGTTGA

Equus caballus transforming growth factor beta 1 (TGFB1), mRNA

NCBI Reference Sequence: NM_001081849.1
>NM_001081849.1 Equus caballus transforming growth factor beta 1 (TGFB1), mRNA
GCGCCGCCTGCCCCATGCCGCCCTCCGGCCTGCGGCTGCTGCCGCTGC
TGCTGCCACTGCTGTGGCTACTAGTGCTGACGCCTGGCCGGCCAGCCG
CCGGACTGTCCACCTGCAAGACCATCGACATGGAGCTGGTGAAGCGG
AAGCGCATCGAGGCCATCCGCGGCCAGATCCTGTCCAAGTTGCGGCTC

GCCAGCCCCCCGAGCCAGGGGGAGGTTCCGCCCGGCCCGCTGCCCGA
GGCCGTGCTGGCCCTTTACAACAGTACCCGCGCCCAGGTGGCCGGAGA
GAGCGCTGAGACGGAGCCCGAGCCTGAGGCGGACTACTACGCCAAGG
AGGTCACCCGCGTGCTAATGGTGGAAAAGGAAAACGAAATCTATAAG
ACTGTGGAGACCGGCTCACACAGCATATATATGTTCTTCAATGCGTCG
GAGCTCCGGGCAGCAGTGCCCGATCCCATGCTGCTCTCCCGGGCAGAG
CTGCGCCTCCTAAGGCTCAAGTTAAGCGTGGAGCAGCATGTGGAGCTG
TACCAGAAATACAGCAATAATTCCTGGCGCTACCTCAGTAACCGGCTG
CTGACCCCCAGCGACTCGCCGGAATGGCTGTCCTTTGATGTCACCGGA
GTCGTGCGGCAGTGGCTGAGCCAGGGAGGGGCAATGGAGGGCCTTCG
CCTCAGTGCCCACTGCCCCTGTGACAGCAAAGATAACACACTCCGCGT
GGGCATCAACGGGTTCAGTTCCAGCCGCCGGGGTGATCTGGCCACCAT
TGATGGCATGAACCGGCCCTTCCTGCTCCTCATGGCCACCCCACTGGA
GAGGGCCCAGCAGCTGCACAGCTCCCGGCACCGCCGAGCTCTGGACA
CCAACTACTGTTCCAGCTCCACAGAGAAGAACTGCTGCGTACGGCAGC
TGTACATTGACTTTCGCAAGGATCTGGGCTGGAAGTGGATCCACGAGC
CCAAGGGCTACCACGCCAACTTCTGCCTGGGGCCCTGCCCCTACATTT
GGAGCCTGGACACGCAGTACAGCAAGGTCCTGGCCCTGTACAACCAG
CACAACCCGGGCGCGTCGGCGGCGCCGTGCTGCGTGCCGCAGGTGCTG
GAGCCGCTGCCCATCGTGTACTACGTGGGTCGCAAGCCCAAGGTGGAG
CAGCTGTCCAACATGATCGTGCGCTCCTGCAAGTGCAGCTGA

Equus caballus interleukin 13 (IL13), mRNA

NCBI Reference Sequence: NM_001143791.1

>NM_001143791.1 Equus caballus interleukin 13 (IL13), mRNA

ATGGCGCTCTGGTTGACAGCAGTCATTGCTCTCGCTTGCCTTGGTGGCC
TTGCCTCCCCAGCCCCTCTGCCATCCTCGATGGCCCTCAAGGAGCTCAT
TAAGGAGCTGGTCAACATCACCCAGAACCAGGCCCCCCTCTGCAATGG
CAGCATGGTGTGGAGCGTCAACCTGACAGCTGACACGTACTGTAGAGC
CCTGGAGTCCCTGAGCAACGTCTCCACCTGCAGTGCCATCCAAAACAC
GCGGAAGATGCTGACTAAACTCTGCCCTCACCAGCTCTCAGCCGGGCA
GGTTTCTAGCGAGCGCGCCCGAGACACCAAAATTGAAGTGATCGTGTT
GGTAAAAGACCTGCTCAAAAATTTAAGGAAAATTTTTCACGGTGGAAA
GCATGTGGACGCCTGA

APPENDIX II SECTION A:

Additional tables and figures / Section B: Published papers

Section A: Additional tables and figures

Appendix II

Section A, Table A1:

Summary table of the cases analysed.

Case No	Uol Ref	horse	Initial Diagnosis	Category	Group	EG /MCT	SEX	Breed	AGE	Location	Morphological Analysis	IHC	ISH
1	05L-4202	1	EG	1	1	EG	Male	Thoroughbred	NA	trunk	Yes	Yes	No
2	05L-0748	2	EG	1	1	EG	NA	NA	NA	NA	Yes	Yes	No
3	05L-1008	3	EG	2	2	MCT	Female	Thoroughbred cross	NA	trunk	Yes	Yes	No
4	05L-1211	4	EG	4	2	MCT	NA	NA	NA	head	Yes	Yes	No
5	05L-1584	5	MCT	4	2	MCT	NA	Thoroughbred	11	leg	Yes	Yes	No
6	05L-2481	6	MCT	3	2	MCT	Male	warmblood	18	NA	Yes	Yes	No
7	05L-2601	7	MCT	5	3	MCT	Female	Arab cross	17	head	Yes	Yes	No
8	06L-0445	8	MCT	4	2	MCT	Male	Thoroughbred cross	12	NA	Yes	Yes	No
9	06L-0445	8	MCT	5	3	MCT	Male	Thoroughbred cross	12	NA	Yes	Yes	No
10	06L-0445	8	MCT	5	3	MCT	Male	Thoroughbred cross	12	NA	Yes	Yes	No
11	06L-0445	8	MCT	5	3	MCT	Male	Thoroughbred cross	12	NA	Yes	Yes	No
12	06L-0445	8	MCT	5	3	MCT	Male	Thoroughbred cross	12	NA	Yes	Yes	No
13	06L-0445	8	MCT	5	3	MCT	Male	Thoroughbred cross	12	NA	Yes	Yes	No
14	06L-0445	8	MCT	5	3	MCT	Male	Thoroughbred cross	12	NA	Yes	Yes	No
15	06L-0445	8	MCT	5	3	MCT	Male	Thoroughbred cross	12	NA	Yes	Yes	No
16	06L-0445	8	MCT	5	3	MCT	Male	Thoroughbred cross	12	NA	Yes	Yes	No
17	06L-1002	9	EG	2	2	MCT	Female	Arab cross	20	head	Yes	Yes	No
18	06L-1257	10	EG	4	2	MCT	Male	Pony	11	NA	Yes	Yes	Yes
19	06L-1943	11	EG	3	2	MCT	Male	Irish draft crossed with Thoroughbred	6	trunk	Yes	Yes	No
20	06L-2715	12	EG	2	2	MCT	Female	Grey Pony	14	head	Yes	Yes	No
21	06L-3482	13	MCT	3	2	MCT	Male	Arab	23	head	Yes	Yes	No
22	06L-3482	13	MCT	2	2	MCT	Male	Arab	23	head	Yes	Yes	No
23	06L-3482	13	MCT	2	2	MCT	Male	Arab	23	head	Yes	Yes	No
24	06L-3482	13	MCT	2	2	MCT	Male	Arab	23	head	Yes	Yes	No
25	06L-3595	14	MCT	3	2	MCT	Male	Thoroughbred Cross	9	NA	Yes	Yes	No
26	06L-3621	15	EG	1	1	EG	Female	NA	8	eyelid/conjunctiva	Yes	Yes	No
27	06L-4011	16	EG	1	1	EG	Male	Thoroughbred cross	12	neck	Yes	Yes	No
28	06L-4554	17	EG	1	1	EG	Female	NA	13	neck/ trunk	Yes	Yes	No
29	06L-4563	18	EG	2	2	MCT	Male	Show Horse	10	NA	Yes	Yes	No

Initial diagnosis *information gathered from diagnostic reports;* ***EG/MCT*** *final diagnosis based on Category grouping: EG (Group1 – Category 1), MCT (Group 2+3 – Categories 2-5); Age: years; Morphological analysis: cases submitted to morphological analysis; IHC: cases submitted to Immunohistochemical analysis (except RANTES). ISH: cases submitted to in situ hybridisation, including IHC for RANTES.*

Case No	Uol Ref	horse	Initial Diagnosis	Category	Group	EG /MCT	SEX	Breed	AGE	Location	Morphological Analysis	IHC	ISH
30	06L-4563	18	EG	5	3	MCT	Male	Show Horse	10	NA	Yes	Yes	No
31	06L-4674	19	MCT	4	2	MCT	Male	Welsh Cob	25	NA	Yes	Yes	No
32	06L-4959	20	EG	1	1	EG	Female	Irish draft crossed with Thoroughbred	7	NA	Yes	Yes	No
33	07L-0586	21	EG	2	2	MCT	Male	Appaloosa	11	NA	Yes	Yes	No
34	07L-2171	22	MCT	4	2	MCT	Male	NA	NA	neck	Yes	Yes	No
35	07L-2900	23	MCT	3	2	MCT	Male	Holstein	12.5	NA	Yes	Yes	No
36	07L-2956	24	EG	3	2	MCT	Female	NA	4	NA	Yes	Yes	No
37	07L-3155	25	EG	1	1	EG	Female	NA	14	NA	Yes	Yes	No
38	07L-3244	26	MCT	5	3	MCT	Male	Arab	21	NA	Yes	Yes	No
39	07L-3335	27	MCT	4	2	MCT	Male	NA	16	eyelied	Yes	Yes	No
40	07L-3494	28	MCT	5	3	MCT	Male	Arab	21	eyelied	Yes	Yes	No
41	07L-3891	29	EG	1	1	EG	Male	Cob	12	NA	Yes	Yes	No
42	08L-0535	30	EG	1	1	EG	Male	Arab	6	NA	Yes	Yes	No
43	08L-1128	31	EG	1	1	EG	Male	Thoroughbred	10	NA	Yes	Yes	No
44	08L-2843	32	MCT	4	2	MCT	Female	Arab	18	leg	Yes	Yes	No
45	08L-3222	33	MCT	4	2	MCT	Female	NA	9	eyelied	Yes	Yes	No
46	08L-3502	34	MCT	3	2	MCT	Male	Arab	3	NA	Yes	Yes	No
47	08L-4509	35	EG	1	1	EG	Male	Thoroughbred	6	NA	Yes	Yes	No
48	09L-0409	36	MCT	4	2	MCT	Female	NA	NA	head	Yes	Yes	No
49	09L-1733	37	EG	2	2	MCT	Female	Warmblood	6	NA	Yes	Yes	No
50	09L-2431	38	EG	1	1	EG	Female	Thoroughbred Cross	NA	NA	Yes	Yes	No
51	09L-2540	39	MCT	4	2	MCT	Male	Thoroughbred Cross	15	head	Yes	Yes	No
52	09L-3044	40	MCT	2	2	MCT	Female	Arab	8	head	Yes	Yes	No
53	09L-3044	40	MCT	3	2	MCT	Female	Arab	8	head	Yes	Yes	No
54	09L-3297	41	EG	1	1	EG	Female	Thoroughbred Cross	11	NA	Yes	Yes	Yes
55	09L-3401	42	EG	4	2	MCT	Female	Thoroughbred	17	head	Yes	Yes	No
56	09L-3661	43	MCT	1	1	EG	Female	Thoroughbred Cross	5	skin	Yes	Yes	No
57	09L-3757	44	EG	2	2	MCT	NA	NA	NA	NA	Yes	Yes	No
58	10L-0007	45	MCT	4	2	MCT	Male	Cob Cross	NA	head	Yes	Yes	No
59	10L-2111	46	MCT	4	2	MCT	Male	French Breed	11	conjunctiva	Yes	Yes	No

Initial diagnosis *information gathered from diagnostic reports;* ***EG/MCT*** *final diagnosis based on Category grouping: EG (Group1 _ Category 1), MCT (Group 2+3 _ Categories 2-5);* ***Age****: years; Morphological analysis: cases submitted to morphological analysis; IHC: cases submitted to Immunohistochemical analysis (except RANTES). ISH: cases submitted to in situ hybridisation, including IHC for RANTES.*

Case No	Uol Ref	horse	Initial Diagnosis	Category	Group	EG /MCT	SEX	Breed	AGE	Location	Morphological Analysis	IHC	ISH
60	10L-3054	47	EG	1	1	EG	Female	Selle Francais	8	NA	Yes	Yes	No
61	10L-3595	48	EG	1	1	EG	Male	Appaloosa	10	eylied	Yes	Yes	No
62	10L-3764	49	MCT	5	2	MCT	Female	Cob	NA	NA	Yes	Yes	No
63	11L-0002	50	EG	1	1	EG	Male	Oldenburg	6	NA	Yes	Yes	No
64	11L-0931	51	EG	1	1	EG	Male	NA	5	NA	Yes	Yes	No
65	11L-2439	52	EG	1	1	EG	Female	Arab	6	NA	Yes	Yes	Yes
66	11L-3186	53	EG	2	2	MCT	Female	NA	NA	NA	Yes	Yes	No
67	11L-3991	54	EG	1	1	EG	Male	New Forest Cross	15	NA	Yes	Yes	Yes
68	11L-4484	55	MCT	4	2	MCT	Male	Welsh Cross	NA	head	Yes	Yes	Yes
69	11L-4484	55	MCT	4	2	MCT	Male	Welsh Cross	NA	head	Yes	Yes	Yes
70	11L-4549	56	MCT	5	3	MCT	Male	Andalusian	10	head	Yes	Yes	No
71	11L-4696	57	MCT	3	2	MCT	Male	NA	3	head	Yes	Yes	Yes
72	11L-4962	58	MCT	3	2	MCT	Male	Welsh cross Thoroughbred	12	head	Yes	Yes	No
73	11L-4962	58	MCT	3	2	MCT	Male	Welsh cross Thoroughbred	12	head	Yes	Yes	No
74	12L-0743	59	EG	2	2	MCT	Female	Appaloosa	12	NA	Yes	Yes	No
75	12L-1868	60	EG	1	1	EG	Male	Irish Sports	5	NA	Yes	Yes	No
76	12l-2837	61	MCT	4	2	MCT	Male	NA	8	head	Yes	Yes	No
77	12L-2922	62	MCT	4	2	MCT	Female	Cohn-X	22	neck	Yes	Yes	No
78	13L-3086	63	EG	1	1	EG	Female	Irish Draught	9	NA	Yes	Yes	No
79	13L-3963	64	EG	1	1	EG	Male	NA	NA	NA	Yes	Yes	No
80	13L-4160	65	EG	1	1	EG	Male	Warmblood	13	NA	Yes	Yes	Yes
81	13L-4160	65	EG	1	1	EG	Male	Warmblood	13	NA	Yes	Yes	No
82	13L-5636	66	MCT	4	2	MCT	Male	NA	8	NA	Yes	Yes	No
83	13L-5690	67	EG	1	1	EG	Male	Welsh Cob	7	NA	Yes	Yes	No
84	13L-5696	68	EG	1	1	EG	Female	Warmblood	6	NA	Yes	Yes	No
85	14L-2190	69	EG	1	1	EG	Male	Arab cross	18	trunk	Yes	Yes	No
86	14L-2190	69	EG	3	2	MCT	Male	Arab cross	18	trunk	Yes	Yes	No
87	14L-3051	70	MCT	4	2	MCT	Male	Welsh cross	8	NA	Yes	Yes	No
88	14L-3456	71	EG	1	1	EG	Male	Spanish	8	trunk	Yes	Yes	No
89	14L-4109	72	EG	1	1	EG	Female	Thoroughbred	6	trunk	Yes	Yes	No

Initial diagnosis *information gathered from diagnostic reports;* ***EG/MCT*** *final diagnosis based on Category grouping: EG (Group1 – Category 1), MCT (Group 2+3 – Categories 2-5);* ***Age****: years; Morphological analysis: cases submitted to morphological analysis; IHC: cases submitted to Immunohistochemical analysis (except RANTES). ISH: cases submitted to in situ hybridisation, including IHC for RANTES.*

Case No	Uol Ref	horse	Initial Diagnosis	Category	Group	EG /MCT	SEX	Breed	AGE	Location	Morphological Analysis	IHC	ISH
90	14L-4723	73	EG	2	2	MCT	Female	Arab	7	trunk	Yes	Yes	No
91	15L-1571	74	MCT	3	2	MCT	Female	Arab	7	NA	Yes	Yes	Yes
92	15L-2513	75	MCT	4	2	MCT	Male	Arab	4	head	Yes	Yes	No
93	15L-2936	76	MCT	4	2	MCT	Male	Welsh cross	13	NA	Yes	Yes	No
94	15L-4057	77	MCT	4	2	MCT	Male	Welsh	10	NA	Yes	Yes	No
95	15L-4903	78	EG	1	1	EG	NA	NA	NA	NA	Yes	Yes	Yes

Initial diagnosis *information gathered from diagnostic reports;* ***EG/MCT*** *final diagnosis based on Category grouping: EG (Group1 – Category 1), MCT (Group 2+3 – Categories 2-5);* ***Age****: years; Morphological analysis: cases submitted to morphological analysis; IHC: cases submitted to Immunohistochemical analysis (except RANTES). ISH: cases submitted to in situ hybridisation, including IHC for RANTES.*

Appendix II

Section A, Table A2:

Summary table of the cases analysed for cytokine/chemokine interaction with raw results.

	Ref.	Categ.	**RANTES**	**Eotaxin**	**MIPα**	**IL4**	**IL5**	**IL13**
1	06L-1257	**IV**	5(5)	0	7	0	340	46
2	11L-4484A	**IV**	7(3)	0 (161*)	2	0	472	88
3	11L-4484B	**IV**	10(4)	0 (94*)	NA	NA	303	39
4	11L-4696	**III**	3(2)	0	7	0	31	5
5	15L-1571a	**III**	8(10)	25	1	NA	85	239
6	09L-3297	**I**	23(16)	4	10	18	28	777
7	11L-2439	**I**	15(9)	2049	10	28	53	362
8	11L-3991	**I**	11(9)	0	29	0	3	431
9	13L-4160a	**I**	14(9)	872	147	21	82	207
10	15L-4903	**I**	7(4)	654	15	0	9	213

Rantes (IHC): mean no cells/40x field (SD). ☐ NA (Not analysed), x 0, x 1-10, x 11-20, x 21-40, x 41-50 cells/40x field. **Rest (ISH)**: total no of positive cells/cm^2. ☐ NA (Not analysed), x 0-10, x 11-100, x 100-500, x 500-1000, x 1000-3500 cells/cm^2.

- ***TGF-β***: Not quantified (constitutively expressed by many cell types in all cases).
- ***SCF/cKIT-L:*** Not quantified (constitutively expressed in all cases by endothelial cells and some vascular smooth muscle cells).
- *****Very Focal Eotaxin expression (not evenly distributed in the lesion). Associated with the presence of focal infiltrates of neutrophils. Considered negative in the whole mass (-), except for those focal areas

Appendix II

Section B: Published papers

Section B: Printout from the publication describing 4 atypical mast cell tumours (*Elbahi A, Kipar A, Ressel L. J Comp Pathol. 2018 July; 162:14-17. doi: 10.1016/j.jcpa.2018.05.003. Epub 2018 June 30*)

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J. Comp. Path. 2018, Vol. 162, 14–17

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NEOPLASTIC DISEASE

Histocytic-like Atypical Mast Cell Tumours in Horses

A. Elbahi[*,‡], A. Kipar[†,‡] and L. Ressel[*,‡]

** Department of Veterinary Pathology and Public Health, Institute of Veterinary Science, University of Liverpool, Leahurst Campus, Chester High Road, Neston, UK, † Institute of Veterinary Pathology, Vetsuisse Faculty, University of Zurich, Winterthurerstrasse 268, Zurich, Switzerland and ‡ Institute of Infection and Global Health, University of Liverpool, Liverpool, Liverpool Science Park IC2, Liverpool, UK*

Summary

This report describes a series of four equine mast cell tumours (MCTs) with atypical morphological features. The tumours were 1–2 cm in diameter and mostly localized to the eyes (one eyelid, two conjunctiva). Histologically, they were composed of very large (up to 35 µm) round pleomorphic cells with a large central to paracentral nucleus and abundant granular cytoplasm. A large number of viable mature eosinophils were detected intermingled with the large round cells. Histochemical staining (toluidine blue and Perls' Prussian blue) and immunohistochemistry (KIT, mast cell tryptase, lysozyme and proliferating cell nuclear antigen) confirmed the mast cell origin of the atypical cells and identified an aberrant KIT protein expression in three cases. Based on morphological and immunohistochemical features, we propose to call the lesions equine histiocytic-like atypical MCTs.

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Keywords: atypical; histiocytic-like; horse; mast cell tumour

Mast cell tumours (MCTs) are uncommon in horses and the majority are benign neoplasms. They are predominantly found in the skin (Valentine, 2006; Scott and Miller, 2011), but have also been described in other locations such as the conjunctiva (Hum and Bowers, 1989). The neoplastic nature of equine MCTs (EMCTs) is still under debate, but there is recent evidence that at least a proportion are truly neoplastic, as they exhibit a high proliferation rate and cellular atypia, in combination with aberrant KIT protein expression (Clarke *et al.*, 2014; Ressel *et al.*, 2015). EMCTs present as nodular masses composed of variable proportions of neoplastic mast cells and mature eosinophils, typically associated with well-circumscribed areas of collagenolysis ('flame figures') that are surrounded by eosinophilic granuloma-like infiltrates (Scott and Miller, 2011; Kiupel, 2017). Cases with low mast cell numbers can present a diagnostic challenge since their morphological features overlap with those of equine eosinophilic granulomas (EEGs) (Kiupel, 2017). On the other hand, in their poorly differentiated form, EMCTs may be difficult to differentiate from other round cell neoplasms (Ressel *et al.*, 2015). The present report describes a series of four EMCTs with morphological and immunohistochemical features similar to those described for atypical histiocytic-like MCTs in cats (Sabattini and Bettini, 2010). We therefore propose to introduce a similar subtype in horses.

A retrospective comparative re-examination of 58 EMCTs and 129 EEGs from the diagnostic archive (years 2005–2015) of the Department of Veterinary Pathology and Public Health, Institute of Veterinary Science, University of Liverpool, identified four lesions with atypical, but similar, features. All four had originally been diagnosed as EEG-like lesions;

Correspondence to: L. Ressel (e-mail: l.ressel@liverpool.ac.uk).

0021-9975/$ - see front matter
https://doi.org/10.1016/j.jcpa.2018.05.003

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[**continued**] Printout from the publication describing 4 atypical mast cell tumours (*Elbahi A, Kipar A, Ressel L. J Comp Pathol. 2018 July; 162:14-17. doi: 10.1016/j.jcpa.2018.05.003. Epub 2018 June 30*)

however, a closer inspection confirmed that they did not exhibit classical features of the latter. Special stains and immunohistochemistry (IHC) had not been performed at the time of the initial diagnosis. Clinically, the lesions had presented as small nodular masses in the haired skin or the conjunctiva (Table 1). Follow-up information was not available.

For re-examination of the four cases, consecutive sections (3–5 μm) were prepared and stained with haematoxylin and eosin (HE), toluidine blue (TB; demonstration of metachromatic mast cell granules) and Perl's Prussian blue (demonstration of haemosiderin). Briefly, for IHC, sections were dewaxed and subjected to antigen retrieval in Dako PT buffer high/low pH (Agilent Technologies Ltd., Stockport, UK) using a computer controlled antigen retrieval workstation (PT Link; Agilent Technologies Ltd.) for 20 min at 98°C. Sections were then labelled in an automated immunostainer (Link 48; Agilent Technologies Ltd.), using primary antibodies against mast cell tryptase (mouse anti-human MCT, clone AA1, Agilent Technologies Ltd.; 1 in 500 dilution), KIT protein (rabbit anti-human CD117 [c-Kit], Agilent Technologies Ltd. [A4502]; 1 in 500 dilution), lysozyme (rabbit anti-human lysozyme, Agilent Technologies Ltd. [A0099]), Iba-1 anti-AIF1/IBA1 antibody (Source Bioscience, Nottingham, UK [LS-B2402]; 1 in 500 dilution) and proliferating cell nuclear antigen (PCNA; mouse anti-PCNA, clone PC10, Dako, Glostrup, Denmark, [M0879]; 1 in 100 dilution) all diluted in EnVisionTM FLEX Antibody Diluent (K8006, Agilent Technologies Ltd.) and tested to cross-react with equine tissues (Ressel *et al.*, 2015) in a 1 h incubation at room temperature (RT). This was followed by a 30 min incubation at RT with the secondary antibody and polymer peroxidase-based detection system (anti-mouse/rabbit Envision Flex+, Agilent Technologies Ltd.). The reaction was 'visualized' with 3, 3′ diaminobenzidine (Agilent Technologies Ltd.). Equine skin with normal mast cells and a lymph node served as positive controls for KIT and MCT as well as Iba-1, lysozyme and PCNA, respectively. Consecutive sections incubated with non-immune rabbit serum or a murine subclass-matched unrelated monoclonal antibody served as negative controls. The positive reaction was represented by a distinct brown cytoplasmic (KIT, Iba-1, MCT, lysozyme, PCNA in mitotic cells), membranous (KIT) or nuclear (PCNA) reaction. The KIT expression pattern was determined according to previously described parameters (Ressel *et al.*, 2015), where a membranous labelling reaction is considered as normal, while cytoplasmic, focally stippled or diffuse labelling is classified as aberrant.

Table 1
Clinical information and gross features of four cases of atypical histiocytic-like mast cell tumour in horses

Case	*Breed*	*Age (years)*	*Tumour location*	*Tumour size (cm)*
1	Thoroughbred	Unknown	Conjunctiva	1.5 × 1.5 × 1
2	Thoroughbred	11	Haired skin (eyelid)	1.5 × 1 × 0.5
3	Thoroughbred	9	Conjunctiva	2 × 1.5 × 0.5
4	Warmblood	4	Haired skin (not specified)	1.5 × 1 × 0.5

Histologically, all four lesions presented as completely excised, well delineated and expansile, densely cellular subepithelial masses. The infiltrates were dominated by viable mature eosinophils, intermingled with individual large (up to 35 μm diameter) pleomorphic round cells with a central to paracentral nucleus, moderate anisokaryosis and anisocytosis and abundant finely granular cytoplasm (Fig. 1). The granular material was strongly metachromatic in the toluidine blue-stained sections (Fig. 1, inset), suggesting their mast cell nature. This was further supported by IHC: the cells showed strong MCT expression (Fig. 2). To obtain further evidence of the neoplastic nature of the large atypical mast cells, the lesions were labelled for KIT and PCNA. In all cases the large cells were KIT positive. Indeed, in 3/4 cases, the KIT expression pattern was that reported for EMCTs suggested to be truly neoplastic (Ressel *et al.*, 2015), representing the aberrant, focally stippled cytoplasmic expression (Fig. 3). They were also mostly PCNA positive (Supplementary Fig. 1), confirming that they were proliferating; however, mitotic figures were rare. In order to fully rule out the initial diagnosis of EEG and considering the size and low nuclear-to-cytoplasmic ratio (both unusual for mast cells), IHC for macrophage markers was performed. This showed that the atypical cells were indeed negative for lysozyme and Iba-1; however, the infiltrates generally contained a moderate number of lysozyme- and Iba-1-positive macrophages (Supplementary Figs. 2 and 3, respectively). Based on their atypical morphology, mast cell tryptase and KIT expression and their proliferative nature, we classified the large cells as neoplastic mast cells and we suggest that the lesions represent equine atypical histiocytic-like mast cell tumours. Supplementary Fig. 4 schematically represents the variability of the neoplastic mast cells in the different EMCT subtypes described so far.

Our case series shares many features with a feline MCT subtype, so-called 'atypical' or 'histiocytic-like' MCTs (Sabattini and Bettini, 2010; Kiupel, 2017). These have been described in cats <4 years of age and are generally small and located within

Appendix II

[**continued**] Printout from the publication describing 4 atypical mast cell tumours (*Elbahi A, Kipar A, Ressel*
L. J Comp Pathol. 2018 July; 162:14-17. doi: 10.1016/j.jcpa.2018.05.003. Epub 2018 June 30)

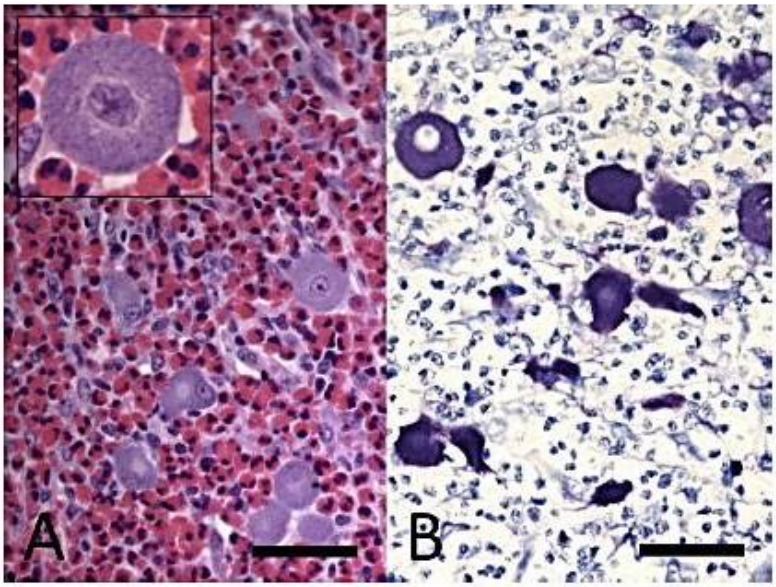

Fig. 1. Case 1. (A) The infiltrate is dominated by viable, mature eosinophils with scattered individual large atypical cells. Inset: atypical cells exhibit abundant granular cytoplasm and round central nuclei with distinct nucleoli. HE. (B) The large cells exhibit abundant metachromatic cytoplasmic granules. Toluidine blue. Bars, 50 µm.

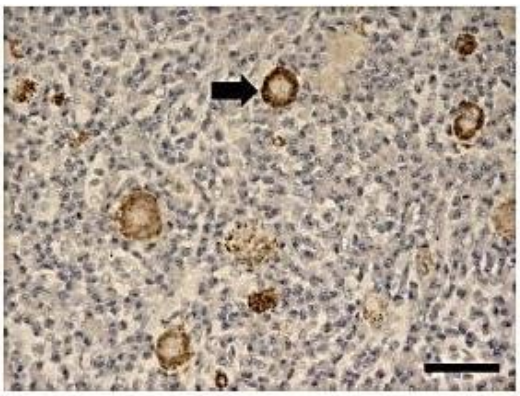

Fig. 2. Case 2. The large atypical cells exhibit strong mast cell tryptase expression (arrow). IHC. Bar, 50 µm.

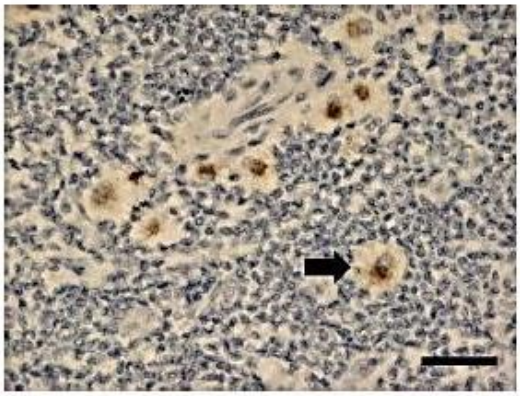

Fig. 3. Case 2. The large atypical cells exhibit a focal stippled cytoplasmic labelling for KIT (arrow). IHC. Bar, 50 µm.

the deep dermis or subcutis (Henry and Herrera, 2013). Histologically, this MCT subtype is characterized by large, polygonal cells with abundant light pink cytoplasm and large nuclei that are found alongside large numbers of viable eosinophils (Sabattini and Bettini, 2010). Due to their histological appearance, feline histiocytic-like MCTs are often misdiagnosed as granulomatous inflammation (Goldschmidt and Hendrick, 2002; Kiupel, 2017). As they are rare, there are insufficient data to assess whether their behaviour differs from that of well-differentiated MCTs in the cat (Kiupel, 2017).

In the present case series, the neoplastic processes were generally small and exhibited features indicative of benign behaviour (i.e. well-demarcated, non-invasive and a very low mitotic rate). However, all cases were lost for any follow-up, so this interpretation cannot be further substantiated; this represents a clear limitation of the study. The processes were predominantly localized around the eye (i.e. conjunctiva

Appendix II

[**continued**] Printout from the publication describing 4 atypical mast cell tumours (*Elbahi A, Kipar A, Ressel L. J Comp Pathol. 2018 July; 162:14-17. doi: 10.1016/j.jcpa.2018.05.003. Epub 2018 June 30*)

and eyelid), both in the mucosa and the haired skin, which raises the question as to whether a specific mast cell subtype is responsible for their particular atypical morphology, or whether the local environment might play a role in the pathogenesis.

In conclusion, the present report describes a morphological variant of EMCTs that has so far not been described. The lesions were histologically different from all previously reported EMCTs and mainly localized to the eyes. Based on their similarity to a subtype of feline MCTs, we propose to call the lesions equine histiocytic-like atypical MCTs.

Acknowledgments

The authors are grateful to the staff of the Histology Laboratory, Veterinary Laboratory Services, Department of Veterinary Pathology and Public Health, University of Liverpool, for excellent technical support. Thanks are due to the clinical colleagues who contributed the cases and retrieved the clinical information. A. Elbahi was supported by a PhD studentship (200948995) from the Libyan Government.

Conflict of Interest Statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

Supplementary Data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jcpa.2018.05.003.

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[*Received, February 18th, 2018*
Accepted, May 25th, 2018]

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